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(54) **L-AMINO ACID-PRODUCING BACTERIUM AND A METHOD FOR PRODUCING L-AMINO ACIDS**

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See application file for complete search history.

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(57) **ABSTRACT**

A method for producing an L-amino acid is provided which includes culturing in a medium a microorganism of the Enterobacteriaceae family which has an ability to produce an L-amino acid and which has been modified so as to enhance the  $\beta$ -glucoside PTS activity, and collecting the L-amino acid from the medium or cells.

**4 Claims, 3 Drawing Sheets**

Fig. 1

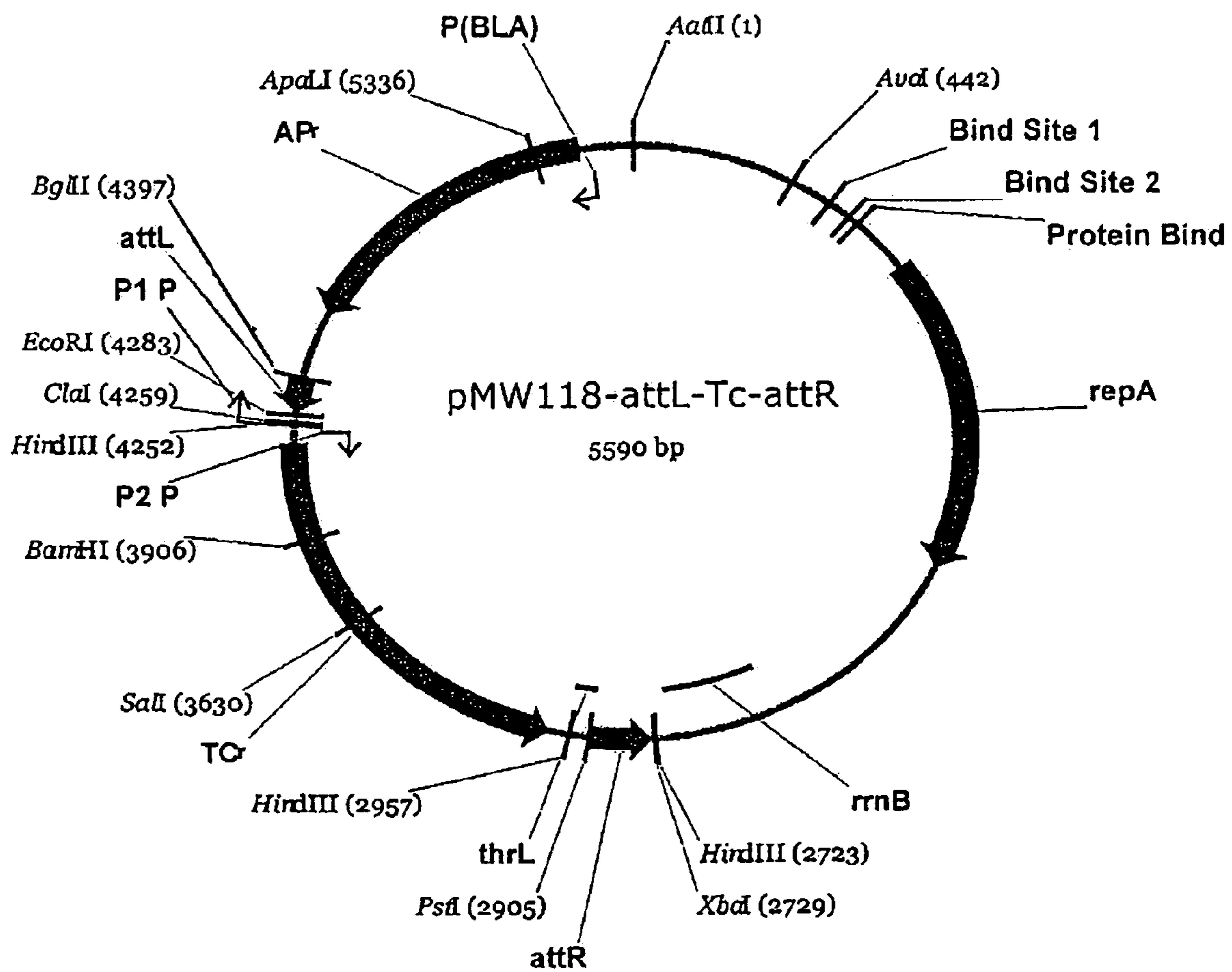


Fig. 2

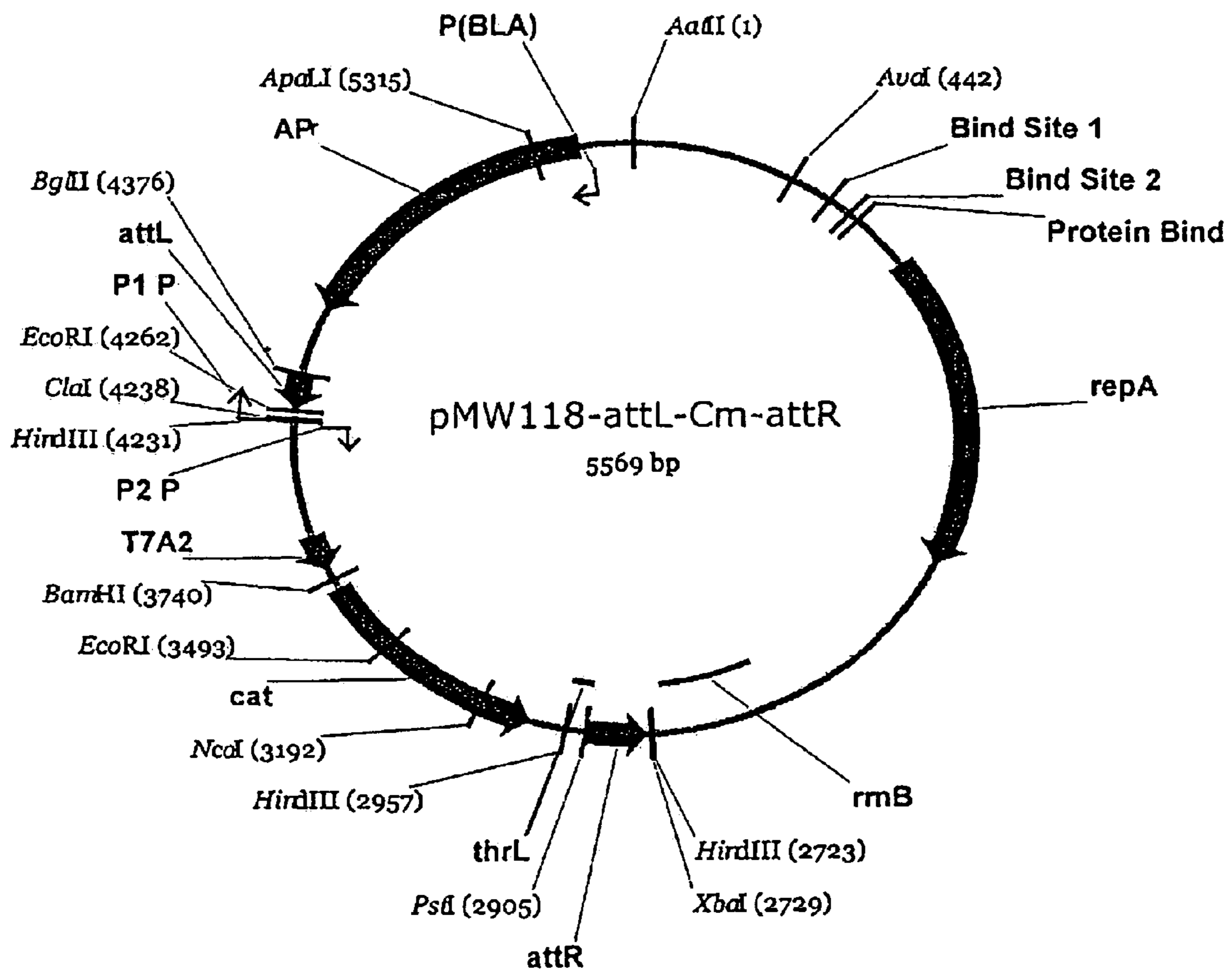
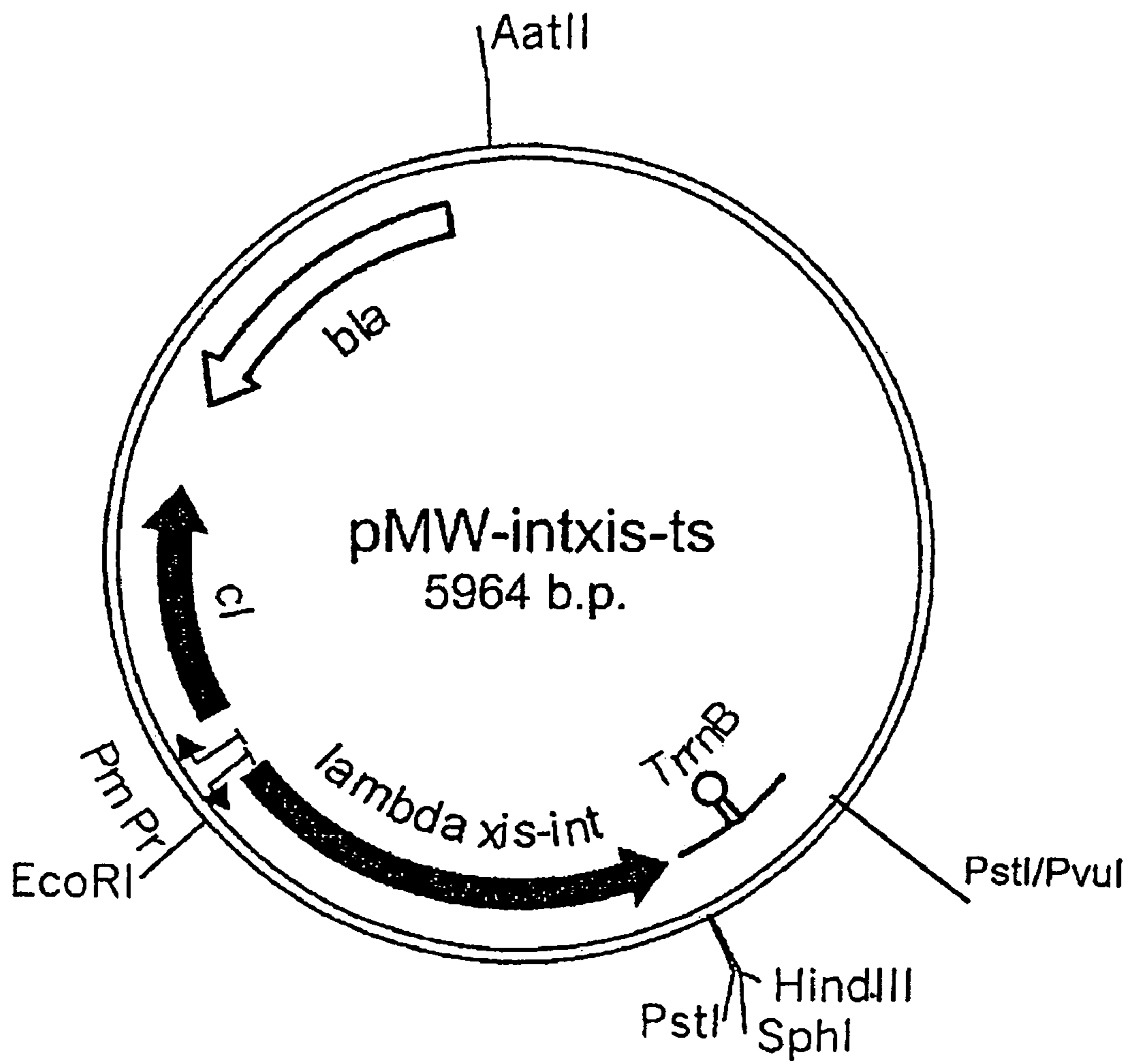


Fig. 3



**L-AMINO ACID-PRODUCING BACTERIUM  
AND A METHOD FOR PRODUCING  
L-AMINO ACIDS**

This application claims priority under 35 U.S.C. §119 to Japanese Patent Application No. 2005-279025, filed on Sep. 27, 2005, U.S. Provisional Patent Application No. 60/723,938, filed on Oct. 6, 2005, and Japanese Patent Application No. 2005-360671, filed on Dec. 14, 2005, and is a continuation application under 35 U.S.C. §120 to PCT Patent Application No. PCT/JP2006/319636, filed on Sep. 25, 2006, the contents of which are incorporated by reference in their entirety. The Sequence Listing filed electronically herewith is also hereby incorporated by reference in its entirety (File Name: US-252\_Seq\_List\_Copy\_1; File Size: 81 KB; Date Created: Mar. 27, 2008).

BACKGROUND OF THE INVENTION

1. Technical Field

The present invention relates to a method for producing an L-amino acid using a microorganism, and more specifically, to a method for producing an L-amino acid, such as L-lysine, L-threonine, and L-glutamic acid, etc. L-lysine and L-threonine are typically used as animal feed additives, health food ingredients, amino acid infusions, etc., and L-glutamic acid is typically used as a seasoning. Therefore, these are industrially useful L-amino acids.

2. Background Art

L-amino acids are industrially produced employing fermentation methods using microorganisms of the genera *Brevibacterium*, *Corynebacterium*, and *Escherichia*, etc. (EP0857784, 0999267, 1170358, JP11-192088A, WO00/53726, WO96/17930, WO03/04674). Wild-type microorganisms, artificial mutants of said bacterial strains, and microorganisms which have been modified so that the activities of the L-amino acid biosynthesis enzymes are enhanced by recombinant DNA techniques are typically used for L-amino acid production.

Known methods for enhancing the ability of various strains to produce an L-amino acid include modifying the L-amino acid uptake or export. For example, to modify the uptake, the ability to produce L-amino acids is enhanced by deleting or reducing the L-amino acid uptake into the cell. For example, one approach is to delete or lower L-glutamic acid uptake by deleting the gluABCD operon or a part of the operon (EP1038970), etc.

One of the methods for modifying the export of an L-amino acid is to delete or reduce the export of an L-amino acid biosynthetic intermediate, and another method is to strengthen the L-amino acid export. For the former, if the target amino acid is L-glutamic acid, reducing the export of  $\alpha$ -ketoglutarate, which is an intermediate in the biosynthesis of L-glutamic acid, by mutating or disrupting the  $\alpha$ -ketoglutarate permease gene has been reported (WO01/005959).

To delete or reduce the export of an L-amino acid biosynthetic intermediate, methods for overexpressing genes responsible for L-amino acid export have been reported, for example, producing L-lysine (WO97/23597) or L-arginine using a bacterial strain of a microorganism of the genus *Corynebacterium* with enhanced expression of the L-lysine or L-arginine export gene (LysE) (Journal of Molecular Microbiology Biotechnology (J Mol Microbiol Biotechnol) 1999 November; 1(2):327-36). Furthermore, increasing the expression of the rhtA, B, and C genes (U.S. Pat. No. 6,303,

348), or the yfiK, yahN genes, etc. has been reported as a method for producing L-amino acids in an *Escherichia* bacteria (EP 1013765).

Aside from modifying the L-amino acid biosynthesis pathway and modifying the uptake and export of the L-amino acid as described above, modifying the ability of the bacteria to take up sugar is another example of a method for improving L-amino acid production. For example, the phosphoenolpyruvate:carbohydrate phosphotransferase system (hereinafter, also referred to as PTS: phosphotransferase) is widely known as a transporter which functions to uptake sugar. Furthermore, PTS is classified as a substrate-independent common system EI (encoded by ptsI), HPr (encoded by ptsH), or substrate-specific component EII. Glucose-specific EII is encoded by ptsG and crr, with the crr gene being a part of an operon with ptsH and ptsI. One known method for producing an L-amino acid uses the genus *Escherichia* in which the ptsG gene has been enhanced (WO03/04670), and another method uses the genus *Escherichia* in which the ptsH, ptsI, and crr genes have been enhanced (WO03/04674).

Aside from the glucose PTS mentioned above, the bglF gene is known to encode  $\beta$ -glucoside specific phosphotransferase (PTS) (Journal of Bacteriology, 1999, Vol. 18, No. 2, p 462-468, Biochemistry, 1998, Vol. 37, p 17040-17047, Biochemistry, 1998, Vol. 37, p 8714-8723), but the use of a gene encoding PTS other than glucose PTS for the production of an L-amino acid has not been reported.

SUMMARY OF THE INVENTION

An aspect of the present invention is to provide a bacterial strain which is capable of efficiently producing an L-amino acid and to also provide a method for producing an L-amino acid using said bacterial strain.

In order to resolve the above-mentioned problem, it has been discovered that an L-amino acid can be effectively produced using a microorganism belonging to the family Enterobacteriaceae which has been modified to increase  $\beta$ -glucoside PTS activity. That is, the present invention is as follows:

It is an aspect of the present invention to provide a method for producing an L-amino acid comprising culturing in a medium a microorganism of the Enterobacteriaceae family which has an ability to produce an L-amino acid and which has been modified to enhance  $\beta$ -glucoside PTS activity as compared to a non-modified microorganism, and collecting the L-amino acid from the medium or cells.

It is an aspect of the present invention to provide the method described above, wherein said  $\beta$ -glucoside PTS activity is enhanced by increasing expression of the bglF gene by a method selected from the group consisting of A) increasing the copy number of the gene, B) modifying an expression regulatory sequence of the gene, and C) combinations thereof.

It is an aspect of the present invention to provide the method described above, wherein said bglF gene is selected from the group consisting of:

(a) a DNA comprising the nucleotide sequence of SEQ ID No. 5,

(b) a DNA encoding a protein having  $\beta$ -glucoside PTS activity which hybridizes with: i) a sequence complementary to nucleotide sequence of SEQ ID No. 5, or ii) a probe prepared from said nucleotide sequence under stringent conditions.

It is an aspect of the present invention to provide the method as described above, wherein the bglF gene encodes a protein selected from the group consisting of: A) a protein comprising the amino acid sequence of SEQ ID NO: 6, and B)

a protein comprising the amino acid sequence of SEQ ID NO: 6, but which includes one or more amino acid substitutions, deletions, additions, or inversions and has  $\beta$ -glucoside PTS activity.

It is an aspect of the present invention to provide the method described above, wherein the microorganism is a bacterium of the genus *Escherichia* or genus *Pantoea*.

It is an aspect of the present invention to provide a method described above, wherein said L-amino acid is selected from a group consisting of L-lysine, L-threonine, L-glutamic acid, and combinations thereof.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the construction of the plasmid pMW118-attL-Tc-attR.

FIG. 2 shows the construction of the plasmid pMW118-attL-Cm-attR.

FIG. 3 shows the construction of the plasmid pMW-intxis-ts.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

Hereinafter, the present invention will be explained in detail.

##### <1> The Microorganism of the Present Invention

The microorganism of the present invention is of the Enterobacteriaceae family and has an ability to produce an L-amino acid. This microorganism also has been modified to enhance the  $\beta$ -glucoside PTS activity. The phrase "an ability to produce an L-amino acid" means the ability to produce and cause accumulation of an L-amino acid in a medium or in the cells of the microorganism when the microorganism of the present invention is cultured in the medium. The microorganism of the present invention may have the ability to produce multiple L-amino acids. The microorganism may inherently possess the ability to produce an L-amino acid, or may be modified by mutagenesis or recombinant DNA techniques to impart the ability to produce an L-amino acid, such as those described below.

The type of L-amino acid is not particularly limited. Examples of the L-amino acid include the basic L-amino acids such as L-lysine, L-ornithine, L-arginine, L-histidine, and L-citrulline; the aliphatic L-amino acids such as L-isoleucine, L-alanine, L-valine, L-leucine, and L-glycine; the hydroxyl L-amino acids such as L-threonine and L-serine; the cyclic L-amino acids such as L-proline; the aromatic L-amino acids such as L-phenylalanine, L-tyrosine, and L-tryptophan; the sulfur-containing L-amino acids such as L-cysteine, L-cystine, and L-methionine; and the acidic L-amino acid such as L-glutamic acid, L-aspartic acid; the amides of acidic L-amino acid such as L-glutamine, L-asparagine, etc. The microorganism of the present invention may have the ability to produce two or more amino acids.

##### <1-1> Imparting L-Amino Acid-Producing Ability

The following examples include a description of the method for imparting L-amino acid-producing ability, along with examples of microorganisms imparted with L-amino acid-producing ability which can be used in the present invention. The microorganisms of the present invention are not limited to these, but can be any as long as they have L-amino acid-producing ability.

There is no particular limitation on the microorganism used in the present invention, as long as it belongs to the family Enterobacteriaceae, such as the genera *Escherichia*, *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia*, *Erwinia*, *Salmo-*

*nella*, *Morganella*, etc., and it has an L-amino acid-producing ability. Specifically, any microorganism belonging to the family Enterobacteriaceae as classified in the NCBI (National Center for Biotechnology Information) database may be used.

It is particularly desirable to use bacteria which belong to the genera *Escherichia*, *Enterobacter*, or *Pantoea* when modifying parent bacterial species.

The parent bacterial strain of the genus *Escherichia* used to obtain the bacteria of the present invention is not particularly limited, but strains listed by Neidhardt et al., may be used (Neidhardt, F. C. et al., *Escherichia coli* and *Salmonella Typhimurium*, American Society for Microbiology, Washington, D.C., 1029 table 1). One example is *Escherichia coli*. Specific examples of *Escherichia coli* are *Escherichia coli* W3110 (ATCC 27325), *Escherichia coli* MG1655 (ATCC 47076), etc., which are prototypes derived from wild-type strains of K12.

These are available, for example, from the American Type Culture Collection (address: P.O. Box 1549 Manassas, Va. 20108, USA). They are available via use of the accession number given to each bacterial strain (see <http://www.atcc.org>). The accession numbers correspond to each bacterial strain, and are listed in the American Type Culture Collection's catalogue.

Examples of bacteria of the genus *Enterobacter* include *Enterobacter agglomerans* and *Enterobacter aerogenes*. An example of a bacterium of the genus *Pantoea* is *Pantoea ananatis*. In recent years, based on 16S rRNA nucleotide sequence analysis, *Enterobacter agglomerans* has on occasion been reclassified as *Pantoea agglomerans*, *Pantoea ananatis*, and *Pantoea stewartii*. For the present invention, any bacterium classified in the family Enterobacteriaceae, whether *Enterobacter* or *Pantoea*, may be employed. The strains *Pantoea ananatis* AJ13355 (FERM BP-6614), AJ13356 (FERM BP-6615), AJ13601 (FERM BP-7207), or any derivative thereof may be employed to breed *Pantoea ananatis* by genetic engineering methods. When isolated, these strains were identified and deposited as *Enterobacter agglomerans*. As stated above, by analysis using the 16S rRNA nucleotide sequence, these bacteria have been reclassified as *Pantoea ananatis*. For the present invention, any bacterium belonging to the genus *Enterobacter* or *Pantoea* may be used as long as the bacterium is classified in the family Enterobacteriaceae.

The following is a description of methods for imparting an L-amino acid-producing ability to a microorganism which belongs to the Enterobacteriaceae family.

To impart the ability to produce an L-amino acid, an auxotrophic mutant, an analog-resistant strain, or a metabolic regulation mutant can be obtained, or a recombinant strain having enhanced expression of an L-amino acid biosynthesis enzyme can be created. Methods conventionally employed in the breeding of coryneform bacteria or bacteria of the genus *Escherichia* (see "Amino Acid Fermentation", Gakkai Shuppan Center (Ltd.), 1st Edition, published May 30, 1986, pp. 77-100) can also be utilized. Here, in the breeding of an L-amino acid-producing bacteria, one or more properties, such as auxotrophic mutation, analog resistance, or metabolic regulation mutation may be imparted. Enhancing the expression of one or more L-amino acid biosynthesis enzymes may also be employed. Furthermore, imparting properties such as auxotrophic mutation, analog resistance, or metabolic regulation mutation may be performed in combination with enhancing the activity of biosynthesis enzymes.

An auxotrophic mutant strain, L-amino acid analog-resistant strain, or metabolic regulation mutant strain with the

ability to produce an L-amino acid can be obtained by subjecting a parent or wild-type strain to a conventional mutation treatment, such as treating with X-rays or UV radiation, or treating with a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine, etc., then selecting those which exhibit an autotrophic mutation, analog resistance, or metabolic regulation mutation and which also have the ability to produce an L-amino acid.

Examples of an L-lysine analog-resistant strain or metabolic regulation mutant include, but are not limited to, the *Escherichia coli* AJ11442 strain (FERM BP-1543, NRRL B-12185, JP56-18596A, and U.S. Pat. No. 4,346,170), and the *Escherichia coli* VL611 strain (EP1016710A), etc. The *Escherichia coli* WC196 strain (WO96/17930) also produces L-lysine. The WC196 strain was bred by imparting AEC (S-(2-aminoethyl)-cysteine) resistance to the W3110 strain derived from *Escherichia coli* K-12. This strain was named *Escherichia coli* AJ13069, and was deposited on Dec. 6, 1994 with the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology (currently, International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology; Chuo 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-8566, Japan) under Accession No. FERM P-14690 and converted to an international deposit under the Budapest Treaty on Sep. 29, 1995, and given Accession No. FERM BP-5252.

L-lysine-producing bacteria can also be constructed by increasing the L-lysine biosynthetic enzyme activity. Examples of genes encoding L-lysine biosynthesis enzymes are the dihydrodipicolinate synthase gene (*dapA*) (EP 0733710B), aspartokinase gene (*lysC*) (EP 0733710, U.S. Pat. No. 5,932,453), dihydrodipicolinate reductase gene (*dapB*), diaminopimelate decarboxylase gene (*lysA*), diaminopimelate dehydrogenase gene (*ddh*) (WO96/40934), the phosphoenolpyruvate carboxylase gene (*ppc*) (JP60-87788A), the aspartate aminotransferase gene (*aspC*) (JP6-102028A), the diaminopimelate epimerase gene (*dapF*) (WO00/56858), the aspartate-semialdehyde dehydrogenase gene (*asd*) (WO00/61723), and other genes of diaminopimelate pathway enzymes; as well as the homoaconitate hydratase gene (JP2000-157276) and other genes of aminoacidipate pathway enzymes. The abbreviations for these genes are given in the parentheses following each name.

Furthermore, it is known that the activities of wild-type dihydrodipicolinate synthase (DDPS) and aspartokinase (AK) are inhibited by feedback by L-lysine; therefore, when *dapA* and *lysC* are used, it is preferable to use genes encoding mutant dihydrodipicolinate synthase and aspartokinase, respectively, that are resistant to the feedback inhibition by L-lysine (EP 0733710, U.S. Pat. No. 5,932,453).

Examples of the DNA encoding mutant dihydrodipicolinate synthase that is resistant to feedback inhibition by L-lysine include a DNA encoding DDPS having an amino acid sequence wherein the 118th histidine residue is substituted with tyrosine. (U.S. Pat. Nos. 5,661,012 and 6,040,160). Furthermore, examples of the DNA encoding a mutant AK that is resistant to feedback inhibition by L-lysine include a DNA encoding AK having the amino acid sequence wherein the 352-threonine residue is substituted with isoleucine. (U.S. Pat. Nos. 5,661,012 and 6,040,160). These mutant DNAs can be obtained by site-directed mutagenesis using PCR, or the like.

The following is an example of imparting an L-lysine-producing ability by introducing a gene encoding an L-lysine biosynthesis enzyme into the host. That is, recombinant DNA is prepared by ligating the gene fragment that encodes the

L-lysine biosynthesis gene with a vector that functions in the host microorganism used in the production of L-lysine, preferably a multi-copy type vector, and this is used to transform the host. By the transformation, the copy number of the gene encoding the L-lysine biosynthesis enzyme in the host cell increases, enhancing the expression and consequently increasing the enzymatic activity.

The genes encoding the L-lysine biosynthesis enzymes are not particularly limited, as long as they can be expressed in the host microorganism. Examples include genes derived from *Escherichia coli*, and genes derived from coryneform bacteria. Because the total genome sequences of *Escherichia coli* and *Corynebacterium glutamicum* have been determined, it is possible to synthesize primers based on the nucleotide sequence of these genes and obtain these genes using the PCR method in which the chromosomal DNA of a microorganism, such as *Escherichia coli* K12, etc., is used as the template.

In order to clone these genes, plasmids that autonomously replicate in the Enterobacteriaceae can be used. Examples include pBR322, pTWV228 (Takara Bio Inc.), pMW119 (Nippon Gene Co., Ltd.), pUC19, pSTV29 (Takara Bio Inc.), RSF110 (Gene vol. 75 (2), pp. 271-288, 1989), etc. In addition, a vector of phage DNA may also be used.

To ligate the target gene to the above-mentioned vector, the vector is digested with a restriction enzyme matched to the end of the DNA fragment containing the target gene. The ligation is usually conducted with a ligase such as T4 DNA ligase. Target genes may be present on separate vectors, respectively, or present on the same vector. Typical methods known to those skilled in the art can be employed for digesting and ligating the DNA, as well as for preparing chromosomal DNA, performing PCR, preparing plasmid DNA, transformation, determining the oligonucleotides for use as primers, etc. These methods are described in Sambrook, J., and Russell, D. W. *Molecular Cloning A Laboratory Manual/Third Edition*. New York: Cold Spring Harbor Laboratory Press (2001), etc. Any method which achieves adequate transformation efficiency may be employed to introduce recombinant DNA that has been prepared as described above into the microorganism. An example includes electroporation (Canadian Journal of Microbiology, 43, 197 (1997)). An example of a plasmid prepared using electroporation is pCABD2, which contains the *dapA*, *dapB*, and *LysC* genes (WO 01/53459).

Enhancing the expression of genes encoding L-lysine biosynthesis enzymes can also be achieved by introducing multiple copies of the target gene into the chromosomal DNA of a microorganism. Multiple copies of the target gene can be introduced into the chromosomal DNA of the microorganism by using a sequence in which multiple copies are present on the chromosomal DNA as a target in homologous recombination. Such site-specific introduction of mutations based on gene substitution using homologous recombination has been described. Methods employing linear DNA or a plasmid containing a temperature-sensitive replication origin have been described (U.S. Pat. Nos. 6,303,383 and 5,616,480). Repetitive DNA and inverted repeats present on the ends of transposable elements can be employed as sequences in which multiple copies are present on chromosomal DNA. An L-lysine biosynthesis gene may be ligated in tandem with a gene which is inherently present on the chromosome, or it may be introduced into a non-essential region on the chromosome or a region of the gene in which the L-lysine yield will be improved if deleted.

Furthermore, as disclosed in U.S. Pat. No. 5,595,889, the target gene may also be located on a transposon, which is then transferred to introduce multiple copies into the chromo-

somal DNA. With either method, the number of copies of the target gene in the transformant increases, so that the enzymatic activity of the L-lysine biosynthesis increases.

In addition to the above-described genetic amplification, an increase in the L-lysine biosynthesis enzyme activity can be achieved by replacing an expression regulatory sequence of the target gene, such as a promoter etc., with a stronger one (see JP1-215280A). For example, the lac promoter, trp promoter, trc promoter, tac promoter, lambda phage PR promoter, PL promoter, and tet promoter are all known as strong promoters. Substitution with these promoters increases expression of the target gene, thus enhancing enzymatic activity. Examples of strong promoters and methods for evaluating the strength of promoters are described in an article by Goldstein et al. (Prokaryotic promoters in biotechnology. *Biotechnol. Annu. Rev.*, 1995, 1, 105-128), etc.

Increasing L-lysine biosynthesis enzyme activity can also be achieved by modifying an element involved in the regulation of the target gene expression, for example, the operator or repressor (Hamilton et al.; *J. Bacteriol.* 1989 September; 171 (9):4617-22). As disclosed in WO 00/18935, a substitution of several bases may be introduced into the -35, -10 region of the promoter of a target gene to modify and strengthen it. Furthermore, substituting several nucleotides into the spacer region between the ribosome binding site (RBS) and the start codon, particularly into the sequence immediately upstream of the start codon, is known to have a strong effect on the mRNA translation efficiency. The expression regulatory regions of the target gene's promoter, etc., can be determined by promoter probe vectors and gene analysis software such as GENETYX, etc. Substitution of expression regulatory sequences can be conducted, for example, in the same manner as in the above-described gene substitution employing temperature-sensitive plasmids. The Red-driven integration method (WO2005/010175) may also used.

Furthermore, in the L-lysine-producing bacteria of the present invention, the activity of an enzyme catalyzing production of a compound other than an L-lysine which branches off from its biosynthesis pathway, or the activity of an enzyme which has a negative effect on the production of L-lysine may be reduced or deleted. These enzymes include homoserine dehydrogenase (thrA), lysine decarboxylase (cadA, lysC), and malic enzyme (sfcA, b2463). The strains with reduced or deficient enzymatic activity are described in WO 95/23864, WO96/17930, WO2005/010175, etc.

To reduce or delete said enzyme activity in a cell, mutagenesis may be performed on the gene which encodes the above-mentioned enzymes, using typical and known methods. This can be achieved, for example, by deleting the gene that encodes the enzyme on the chromosome using genetic recombination, or by modifying the expression regulatory sequence of a promoter or a Shine-Delgarno (SD) sequence, etc. This can also be achieved by introducing an amino acid substitution (missense mutation) or stop codon (nonsense mutation) in the region encoding the enzyme on the chromosome, by introducing a frameshift mutation to add or delete 1-2 bases, or by deleting a part of the gene or the entire region (*Journal of Biological Chemistry* 272:8611-8617 (1997); *Journal of Antimicrobial Chemotherapy* 200 46, 793-796; *Biotechnol Prog* 1999, 15, 58-64; *J. Biological Chemistry* vol 272 NO. 13 pp 8611-8617). Also, the enzyme activity can be reduced or deleted by constructing a gene that encodes the mutant enzyme in which the encoded region has been deleted and then substituting the wild-type gene on the chromosome with this, by homologous recombination, etc., or introducing a transposon or IS element into said gene.

The following methods may be used to introduce a mutation which reduces or deletes the above-mentioned enzyme activity by genetic recombination. An isolated DNA containing the target gene is mutated so that the resulting mutant gene does not produce an enzyme that functions normally. Then, transforming this into a microorganism which belongs to the family Enterobacteriaceae using the DNA containing the gene, and generating the recombination of the mutant-type gene with a gene on the chromosome. For gene substitution using this kind of homologous recombination, there are methods which employ linear DNA, such as the method called "Red-driven integration" (*Proc. Natl. Acad. Sci. USA*, 2000, vol. 97, No. 12, pp. 6640-6645), or by combining the Red-driven integration method and the  $\lambda$  phage excisive system (*J. Bacteriol.* 2002 September; 184 (18): 5200-3, Interactions between integrase and excisionase in the phage lambda excisive nucleoprotein complex. Cho E H, Gumport R I, Gardner JF) (see WO2005/010175), etc.; and there are methods which employ a plasmid containing a temperature-sensitive replication origin (*Proc. Natl. Acad. Sci. USA*, 2000, vol. 97, No. 12, pp. 6640-6645, U.S. Pat. Nos. 6,303,383, or 5,616,480). Such site-specific introduction of mutations via gene substitution using homologous recombination as described above may also be performed using a plasmid which does not have replication ability in the host.

The above-mentioned method for increasing the enzyme activity involving L-lysine biosynthesis and the method for lowering the enzyme activity may likewise be used in breeding other L-amino acid-producing bacteria. The following is a description of methods for breeding other L-amino acid bacteria.

As the L-glutamic acid-producing bacteria used in the present invention, there is, for example, a microorganism which belongs to the family Enterobacteriaceae which has been modified to increase the expression of a gene encoding an enzyme that is involved in L-glutamic acid biosynthesis. The enzymes involved in L-glutamic acid biosynthesis include glutamate dehydrogenase (gdh), glutamine synthetase (gltAB), glutamate synthase (glnA), isocitrate dehydrogenase (icd), aconitate hydratase (acn), citrate synthase (gltA), phosphoenolpyruvate carboxylase (ppc), pyruvate carboxylase (pycA), pyruvate dehydrogenase (pdhA), pyruvate kinase (pykA), phosphoenolpyruvate synthase (pps), enolase (eno), phosphoglucosmutase (pgm), phosphoglycerate kinase (pgk), glyceraldehyde-3-phosphate dehydrogenase (gpd), triose phosphate isomerase (tpi), fructose-bisphosphate aldolase (fba), phosphofructokinase (pfk), glucosephosphate isomerase (gpi), etc. Of these enzymes, citrate synthase, phosphoenolpyruvate carboxylase, glutamate dehydrogenase, and combinations thereof are preferable, and the use of all three is more preferable.

Examples of microorganisms belonging to the family Enterobacteriaceae which have been modified to enhance the expression of the citrate synthase gene, phosphoenolpyruvate carboxylase gene, and/or glutamate dehydrogenase gene using the methods described above are described in U.S. Pat. Nos. 6,197,559 & 6,331,419, EP0999282, and WO2006/051660.

Furthermore, microorganisms belonging to the family Enterobacteriaceae which have been modified to increase the activity of either 6-phosphogluconate dehydratase or 2-keto-3-deoxy-6-phosphogluconate aldolase, or both, may also be used. (EP1352966B)

The microorganisms of the family Enterobacteriaceae having the ability to produce an L-glutamic acid which may be used include a bacterium in which the activity of an enzyme that catalyzes production of a compound other than



L-glutamic acid, but which branches off from the biosynthesis pathway of L-glutamic acid, has been reduced or lowered. Examples of such enzymes include 2-oxoglutarate dehydrogenase (sucA), isocitrate lyase (aceA), acetohydroxy acid synthase (ilvG), acetolactate synthase (ilvN), formate acetyltransferase (pflB), lactate dehydrogenase (ldh), glutamate decarboxylase (gadA), and 1-pyrroline dehydrogenase (putA), etc. Of these, it is especially preferable to reduce or delete the activity of 2-oxoglutarate dehydrogenase.

Methods for deleting or reducing the activity of 2-oxoglutarate dehydrogenase in a microorganism belonging to the family Enterobacteriaceae are described in U.S. Pat. Nos. 5,573,945, 6,197,559, and 6,331,419. Examples of microorganisms belonging to the family Enterobacteriaceae wherein the activity of 2-oxoglutarate dehydrogenase has been deleted or reduced include the following:

*Pantoea ananatis* AJ13601 (FERM BP-7207)

*Klebsiella planticola* AJ13410 strain (FERM BP-6617)

*Escherichia coli* AJ12949 (FERM BP-4881), and others.

The AJ12949 strain has reduced  $\alpha$ -ketoglutarate dehydrogenase activity, and was deposited on Dec. 28, 1993 with the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology (currently, International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology; Chuo 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-8566, Japan) under Accession No. FERM P-14039 and converted to an international deposit under the Budapest Treaty on Nov. 11, 1994, and given Accession No. FERM BP-4881.

The L-tryptophan-producing bacteria preferably used in the present invention are bacteria in which the activity of one or more of the following enzymes, i.e., anthranilate synthase (trpE), phosphoglycerate dehydrogenase (serA), or tryptophan synthase (trpAB) has been enhanced. Since anthranilate synthase and phosphoglycerate dehydrogenase both are subject to feedback inhibition by L-tryptophan and L-serine, the activities of these enzymes can be increased by retaining the desensitizing mutant enzyme (U.S. Pat. Nos. 5,618,716, 6,180,373). For instance, it is possible to obtain bacteria which have a desensitizing enzyme by mutating the anthranilate synthase gene (trpE) and/or the phosphoglycerate dehydrogenase gene (serA) to prevent feedback inhibition, then introducing the mutant gene into a microorganism belonging to the family Enterobacteriaceae. A specific example of this kind of bacteria is *Escherichia coli* SV164 which retains desensitized anthranilate synthase and which has been transformed with plasmid pGH5 having a mutated serA that encodes desensitized phosphoglycerate dehydrogenase (WO94/08301).

Bacteria transformed with recombinant DNA containing a tryptophan operon are also preferable L-tryptophan-producing bacteria. A specific example is *Escherichia coli* transformed with a tryptophan operon containing a gene encoding desensitized anthranilate synthase (trpAB) (Japanese Patent Application Publication No. JP57-71397, Japanese Patent Application Publication No. JP 62-244382, U.S. Pat. No. 4,371,614). Furthermore, in the tryptophan operon, it is possible to enhance the ability to produce L-tryptophan by increasing the expression of the gene (trpBA) encoding tryptophan synthase. Tryptophan synthase contains  $\alpha$  and  $\beta$  subunits that are encoded by trpA and trpB, respectively (WO2005/103275).

Examples of L-tryptophan-producing bacteria are *Escherichia coli* AGX17 (pGX44) [NRRL B-12263], which requires L-phenylalanine and L-tyrosine for growth, and AGX6

(pGX50) aroP [NRRL B-12264], which retains plasmid pGX50 containing a tryptophan operon (see U.S. Pat. No. 4,371,614).

A strain with a deficient tryptophan operon repressor (trpR), and a strain with a mutant trpT are also desirable L-tryptophan-producing bacteria. (U.S. Pat. No. 4,371,614 WO2005/056776).

Another preferable L-tryptophan-producing bacterium is the bacterium in which malate synthase (aceB), isocitrate lyase (aceA), and the isocitrate dehydrogenase/phosphatase (icl) operon (ace operon) are structurally expressed, or the expression of said operon has been enhanced (WO2005/103275).

L-tryptophan, L-phenylalanine, and L-tyrosine are all aromatic amino acids and share a biosynthesis system. Examples of genes encoding biosynthesis enzymes of aromatic amino acids include deoxyarabino-heptulosonate phosphate synthase (aroG), 3-dehydroquinate synthase (aroB), shikimate dehydratase, shikimate kinase (aroL), 5-enolpyruvylshikimate[ $\beta$ ]-phosphate synthase (aroA), and chorismate synthase (aroC) (European Patent Application Publication No. 763127). Therefore, by placing multiple copies of the genes encoding these enzymes onto a plasmid or genome, the aromatic amino acid-producing ability can be improved. It is known that these genes are controlled by a tyrosine repressor (tyrR), so the biosynthesis enzyme activity of an aromatic amino acid may also be increased by deleting the tyrR gene (EP763127).

The L-threonine-producing bacteria are preferably microorganisms belonging to the family Enterobacteriaceae wherein the L-threonine biosynthesis enzymes have been enhanced. Examples of genes encoding L-threonine biosynthesis enzymes include the aspartokinase III gene (lysC), the aspartate-semialdehyde dehydrogenase gene (asd), the aspartokinase I gene encoding the thr operon (thrA), the homoserine kinase gene (thrB), and the threonine synthase gene (thrC). The abbreviations for these genes are given in parentheses following their names. One or more of these genes may be introduced. The L-threonine biosynthesis gene may be introduced into a bacterium of the genus *Escherichia* wherein threonine degradation has been suppressed. Examples of bacteria of the genus *Escherichia* wherein threonine degradation has been suppressed include the TDH6 strain wherein the threonine dehydrogenase activity has been deleted (Japanese Patent Application Publication No. 2001-346578), and so forth.

Activities of some of the L-threonine biosynthesis enzymes are suppressed by the L-threonine that is produced. Therefore, in order to construct an L-threonine-producing bacterium, it is preferable to modify the L-threonine biosynthesis enzyme so that the enzyme is not subject to feedback inhibition by L-threonine. The above-mentioned thrA, thrB, and thrC genes make up the threonine operon, which is in the form of an attenuator structure. The expression of the threonine operon is subject to inhibition by isoleucine and threonine present in the culture, and the expression is attenuated. This modification of the threonine operon can be achieved by removing the leader sequence in the attenuation region or the attenuator. (WO 02/26993; Biotechnology Letters Vol. 24, No. 21, November 2002; WO2005/049808).

A native promoter is located on the threonine operon, and may be substituted with a non-native promoter (WO 98/04715). Alternatively, a threonine operon may be constructed so that the expression of the gene involved in threonine biosynthesis is controlled by a lambda phage repressor and promoter. (EP0593792). Also, to prevent feedback inhibition by L-threonine, modification of the bacteria of the

genus *Escherichia* can also be obtained by selecting an  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid (AHV) resistant bacterial strain (JP45026708B).

It is preferred that the copy number of the threonine operon which is modified to prevent feedback inhibition by L-threonine is increased in the host or is ligated to a strong promoter. In addition to amplifying the copy number of the gene using a plasmid, the copy number of the gene can be increased by introducing the threonine operon onto the chromosome using a transposon, Mu-phage, etc.

For the aspartokinase III gene (*lysC*), it is desirable to use a gene modified to prevent feedback inhibition by L-lysine. A *lysC* gene which has been modified to prevent feedback inhibition can be obtained using the method described in the U.S. Pat. No. 5,932,453.

Aside from the L-threonine biosynthesis enzyme, it is desirable to strengthen genes involved in the glycolytic system, TCA cycle, and respiratory chain, a gene which controls gene expression, and a gene which induces uptake of sugar. Examples of these genes which are effective in L-threonine production include the transhydrogenase gene (*pntAB*) (EP733712), phosphoenolpyruvate carboxylase gene (*ppc*) (WO 95/06114), the phosphoenolpyruvate synthase gene (*pps*) (EP 877090), and the pyruvate carboxylase gene in the coryneform bacteria or *Bacillus* bacteria (WO99/18228, EP1092776).

It is also preferable to enhance the expression of a gene that imparts resistance to L-threonine and a gene that imparts resistance to L-homoserine, or to impart both L-threonine resistance and L-homoserine resistance to the host. Examples of such genes are the *rhtA* gene (Res Microbiol. 2003 March; 154 (2): 123-35), the *rhtB* gene (EP0994190), the *rhtC* gene (EP1013765), the *yfiK* gene, and the *yeaS* gene (EP1016710). To impart L-threonine resistance to a host, refer to European Patent Application Publication No. 0994190 and WO 90/04636.

Another example of an L-threonine-producing bacterium is the *Escherichia coli* VKPM B-3996 strain (U.S. Pat. No. 5,175,107). This VKPM B-3996 strain was deposited on Nov. 19, 1987, under Accession No. VKPM B-3996, at the Russian National Collection of Industrial Microorganisms (VKPM), GNII Genetika. In addition, the VKPM B-3996 strain retains plasmid pVIC40 (WO90/04636) obtained by inserting a threonine biosynthesis gene (threonine operon: *thrABC*) into a wide-host vector plasmid pAY32 including a streptomycin-resistant marker (Chistorerdov, A. Y., Tsygankov, Y. D., Plasmid, 1986, 16, 161-167). In this pVIC40, the feedback inhibition by the L-threonine of the aspartokinase I-homoserine dehydrogenase I that the *thrA* in the threonine operon encodes has been desensitized.

A further example is the *Escherichia coli* B-5318 strain (see European Patent No. 0593792). The B-5318 strain was deposited under Accession No. VKPM B-5318 at the Russian National Collection of Industrial Microorganisms (VKPM), GNII Genetika (Russia, 117545 Moscow, 1 Dorozhny Proezd, 1) on May 3, 1990. This VKPM B-5318 strain is an isoleucine non-auxotrophic strain, and retains recombinant plasmid DNA constructed in such a way that the gene involved in threonine biosynthesis, i.e., the threonine operon wherein the attenuator region and the native transcriptional regulatory region has been deleted, is located downstream of the lambda phage temperature-sensitive CI repressor, PR promoter, and the N-terminus of Cro protein of lambda phage, and expression of the gene involved in the threonine biosynthesis is controlled by the lambda phage repressor and promoter.

Examples of preferred L-histidine-producing strains include the *Escherichia coli* FERM P-5038 and 5048 strains harboring vectors in which genetic information involved in L-histidine biosynthesis have been incorporated (JP56-005099A), a bacterial strain into which the amino acid export gene *Rht* has been introduced (EP1016710), and the *Escherichia coli* 80 strain which has resistance to sulfaguanidine, D, L-1,2,4-triazole-3-alanine, and streptomycin (VKPM B-7270, Russian Patent Publication No. 2119536), etc.

Microorganisms in which expression of the gene encoding the L-histidine biosynthesis pathway enzyme may be used to produce L-histidine. Examples of L-histidine biosynthesis enzymes are ATP phosphoribosyltransferase (*hisG*), phosphoribosyl AMP cyclohydrolase (*hisI*), phosphoribosyl-ATP pyrophosphohydrolase (*hisIE*), phosphoribosylformimino-5-aminoimidazole carboxamide ribotide Isomerase (*hisA*), amidotransferase (*hisH*), histidinol phosphate aminotransferase gene (*hisC*), histidinol phosphatase gene (*hisB*), and histidinol dehydrogenase gene (*hisD*), etc.

The preferred L-cysteine-producing bacteria of the present invention are bacteria in which the activity of the cystathionine  $\beta$ -lyase has been reduced (JP2003-169668), and bacteria of the genus *Escherichia* that retain serine acetyltransferase with reduced feedback inhibition by L-cysteine (JP11-155571).

The preferred L-proline-producing bacteria of the present invention include *Escherichia coli* 702 (VKPMB-8011) which is resistant to 3,4-dehydroxyproline and azetidine-2-carboxylate, and 702 *ilvA* (VKPMB-8012 strain), which is deficient in *ilvA*, and is derived from 702 (JP 2002-300874A).

Examples of L-phenylalanine-producing bacteria include AJ12739 (*tyrA::Tn10, tyrR*) (VKPM B-8197) which is deficient in *tyrA* and *tyrR*, and strains with amplified genes encoding phenylalanine export proteins, such as *yddG* and *yedA*.

Examples of L-arginine-producing bacteria include *Escherichia coli* mutant strains which are resistant to  $\alpha$ -methylmethionine, p-fluorophenylalanine, D-arginine, arginine hydroxamic acid, S-(2-aminoethyl)-cysteine,  $\alpha$ -methylserine,  $\beta$ -2-thienylalanine, or sulfaguanidine (JP56-106598), etc. The *Escherichia coli* 237 strain is an L-arginine-producing bacterium that has a mutant which is resistant to feedback inhibition by L-arginine and that retains highly active N-acetyl glutamate synthase, and it is also a preferable L-arginine-producing strain. (EP1170361B). This strain, numbered VKPM B-7925, was deposited with the Russian National Collection of Industrial Microorganisms (VKPM), GNII Genetika on Apr. 10, 2000, and converted to an international deposit under the Budapest Treaty on May 18, 2001. The *Escherichia coli* 382 strain, which is a derivative of the 237 strain and is an L-arginine-producing bacterium with improved acetic acid assimilating ability, may also be used (U.S. Pat. No. 6,841,365). The *Escherichia coli* 382 strain, numbered VKPM B-7926, was deposited with the Russian National Collection of Industrial Microorganisms (VKPM) on Apr. 10, 2000.

Also, as the microorganisms having L-arginine-producing ability, microorganisms with improved expression of genes encoding enzymes involved in L-arginine biosynthesis may be used. Examples of L-arginine biosynthesis enzymes include N-acetyl glutamate synthase (*argA*), N-acetylglutamyl-phosphate reductase (*argC*), ornithine acetyltransferase (*argJ*), N-acetyl glutamate kinase (*argB*), acetyl ornithine transaminase (*argD*), acetyl ornithine deacetylase (*argE*), ornithine carbamoyl transferase (*argF*), argininosuccinate synthase (*argG*), argininosuccinate lyase (*argH*), car-

bamoyl phosphate synthase (*carAB*), and combinations thereof. After each enzyme name, the name of the gene encoding it is given in parentheses. It is desirable to employ a mutation of the N-acetyl glutamate synthase gene (*argA*) in which L-arginine feedback inhibition has been removed by substitution of the amino acid sequence corresponding to positions 15 to 19 in the wild-type (EP EP1170361).

The L-leucine-producing bacteria which may be used include a bacterium of the genus *Escherichia coli* in which the branched-chain amino-acid transaminase encoded by the *ilvE* gene has been inactivated and the activity of the aromatic amino acid transaminase encoded by the *tyrB* gene has been enhanced (EP1375655A), the *Escherichia coli* H-9068 strain (ATCC21530) which is resistant to 4-azaleucine or 5,5,5-trifluoro-leucine, the *Escherichia coli* H-9070 strain (FERM BP-4704), the *Escherichia coli* H-9072 strain (FERM BP-4706) (U.S. Pat. No. 5,744,331), the *Escherichia coli* strain in which the isopropylmalate synthase feedback inhibition by L-leucine has been desensitized (European Patent No. 1067191), the *Escherichia coli* AJ11478 strain which is resistant to  $\beta$ -2 thienylalanine and  $\beta$ -hydroxyleucine (U.S. Pat. No. 5,763,231), and so on.

L-isoleucine-producing bacteria include a 6-dimethyl aminopurine-resistant *Escherichia coli* mutant strain (JP 5-304969A), L-isoleucine hydroxamate-resistant *Escherichia coli* mutant strain (JP5-130882A), thiaisoleucine-resistant *Escherichia coli* mutant strain (JP5-130882A), DL-ethionine-resistant *Escherichia coli* mutant strain (JP5-130882A), and arginine hydroxamate-resistant mutant strain (JP5-130882A), all of which have L-isoleucine-producing ability. Examples of recombinant bacteria of the genus *Escherichia* are bacterial strains in which the expression of the genes encoding the L-isoleucine biosynthesis enzymes threonine deaminase or acetohydroxy acid synthase have been increased (JP2-458A, JP2-42988A, JP 8-47397A), etc.

Examples of parent strains for deriving L-valine-producing bacteria of the present invention include, but are not limited to, strains which have been modified to overexpress the *ilvGMEDA* operon (U.S. Pat. No. 5,998,178). It is desirable to remove the region in the *ilvGMEDA* operon which is required for attenuation so that expression of the operon is not attenuated by L-valine. Furthermore, the *ilvA* gene in the operon is desirably disrupted to decrease threonine deaminase activity.

Examples of parent strains for deriving L-valine-producing bacteria of the present invention include mutants having a mutation in the amino-acyl t-RNA synthetase (U.S. Pat. No. 5,658,766). For example, *E. coli* VL1970, which has a mutation in the *ileS* gene encoding isoleucine tRNA synthetase, can be used. *E. coli* VL1970 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 113545 Moscow, 1 Dorozhny Proezd, 1) on Jun. 24, 1988 under accession number VKPM B-4411.

Furthermore, mutants requiring lipoic acid for growth and/or lacking H<sup>+</sup>-ATPase can also be used as parent strains (WO96/06926).

Aside from a gene which encodes a native biosynthesis enzyme, a gene which is involved in sugar uptake, sugar metabolism (glycolytic system), and energy metabolism may be enhanced in the L-amino acid-producing bacteria of the present invention.

Examples of the genes involved in sugar metabolism are genes which encode glycolytic enzymes or proteins which uptake sugar, such as genes encoding the glucose-6-phosphate isomerase gene (*pgi*; WO01/02542), the phosphoenolpyruvate synthase gene (*pps*), the phosphoglucosylase gene (*pgm*; WO03/04598), the fructose-bisphosphate aldo-

lase gene (*fba*; WO03/04664), the pyruvate kinase gene (*pykF*; WO03/008609), the transaldolase gene (*talB*; WO03/008611), the fumarase gene (*fum*; WO01/02545), the phosphoenolpyruvate synthase gene (*pps*; EP877090), the non-PTS sucrose uptake systems gene (*csc*; EP149911), and the sucrose-assimilating genes (*scrAB* operon; WO90/04636).

Examples of the genes involved in energy metabolism include the transhydrogenase gene (*pntAB*; U.S. Pat. No. 5,830,716) and the cytochrome bo type oxidase gene (*cyoABCD*; EP1070376).

<1-2> Method for Increasing the Activity of  $\beta$ -Glucoside PTS

The microorganism of the present invention can be obtained by modifying a microorganism which has the ability to produce an L-amino acid and which belongs to the Enterobacteriaceae family, as described above, so as to increase the enzymatic activity of the  $\beta$ -glucoside PTS. However, the ability to produce an L-amino acid may be imparted after modification to increase the enzymatic activity of the  $\beta$ -glucoside PTS.

An increase in the enzymatic activity of the  $\beta$ -glucoside PTS can be achieved by modifying the expression of the *bglF* gene which encodes the  $\beta$ -glucoside PTS (described later). The expression of the endogenous *bglF* gene may be increased through modification of the expression regulatory region, including promoter modification, or the expression of the exogenous *bglF* gene may be increased by introduction of a plasmid containing the *bglF* gene, increasing the number of copies by amplifying the *bglF* gene on the chromosome, etc. Furthermore, a combination of these techniques may be employed.

The  $\beta$ -glucoside PTS in the present invention refers to a permease activity which results in uptake of sugar into the cytoplasm at the same time that the phosphate group in phosphoenolpyruvate (hereinafter, referred to as PEP) is transferred to the  $\beta$ -glucoside. Here, the  $\beta$ -glucoside has 1-D-glucose as the sugar component, for instance, salicin which has been glucoside-linked with salicyl alcohol, or arbutin which has been glucoside-linked to hydroquinone, and generally means a sugar derivative in which various compounds, such as alcohol, phenol, anthocyanin, etc., have been linked to the reduction group of the  $\beta$ -D-glucose. The  $\beta$ -glucoside PTS may also function to transfer the phosphate group, not only to the  $\beta$ -glucoside, but also to the glucose at the same time (*E. coli* & *Salmonella* 2nd Edition American society for Microbiology).

An increase in the enzymatic activity of the  $\beta$ -glucoside PTS can be confirmed by in vitro measurement of the phosphorylating activity, using the method of Chen et al. (Biochemistry 1998 37:8714-8723) (EC 2.7.1.69). Enhancement of the expression of *bglF* can also be confirmed by comparing the amounts of mRNA of *bglF* with that in a wild-type or non-modified strain of bacteria. Northern hybridization and RT-PCR can also be used to confirm expression. (Molecular Cloning (Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001)). The degree of increase in enzymatic activity is not limited as long as the activity is increased as compared to that in the wild-type or non-modified strain, but it is desirable, for example, for it to be 1.5 or more times, preferably 2 or more times, or more preferably 3 or more times that of the wild or non-modified strain. An increase in the enzymatic activity can be confirmed if the target protein amount is increased relative to that of the non-modified or wild-type strain. This can be detected, for instance, by Western blot using an antibody (Molecular Cloning (Cold Spring Harbor Laboratory Press, Cold spring Harbor (USA), 2001)). The *bglF* gene of the present invention is derived from or

native to the bacteria of the genus *Escherichia* and their homologs. For example, the bglF gene of *Escherichia coli* encodes a protein with the amino acid sequence of SEQ ID No. 6. The gene is registered with Genbank NP\_418178 and W3110's sequence is registered with Genbank PTV3B\_ECOLI [P08722], both are identical to SEQ ID NO.5. The bglF gene of *Escherichia Coli* is shown in SEQ ID No.5, and the amino acid sequence is shown in SEQ ID No. 6.

The homologs of the bglF gene include those which are derived from or native to other microorganisms, and which have high similarity in structure to the bglF gene of *Escherichia coli*, and which improve the ability to produce an L-amino acid and exhibit  $\beta$ -glucoside PTS activity when introduced into a host. Examples of bglF homologs are the bglF gene from *Erwinia carotovora* (Genbank Accession No. YP\_050260), the bglF gene from *Streptococcus agalactiae* (NP-735260), and the bglF gene from *Photobacterium luminescens* subsp. (NP\_927931). Furthermore, based on the homology with the genes given in the above examples, the bglF gene may be cloned from the coryneform group of bacteria, such as *Corynebacterium glutamicum*, *Brevibacterium lactofermentum*, etc.; the bacteria of the genus *Pseudomonas*, such as *Pseudomonas aeruginosa*, etc.; the bacteria of the genus *Mycobacterium*, such as *Mycobacterium tuberculosis*, etc.; and so forth. For example, the bglF gene may be cloned using synthetic oligonucleotides SEQ ID Nos. 1 and 2.

The genes encoding the  $\beta$ -glucoside PTS used in the present invention are not limited to the wild-type genes, and as long as the function of the encoded  $\beta$ -glucoside PTS protein, i.e., the  $\beta$ -glucoside PTS activity, is not impaired. They can also be a mutant or an artificially modified product encoding a protein which includes a sequence containing several amino acid substitutions, deletions, insertions, additions, or the like at one or multiple positions in the amino acid sequence of SEQ ID No. 6. Here, the term "several" varies with the type and position of the amino acid residues in the stereostructure of the protein. Specifically, it means 1 to 20, preferably 1 to 10, and more preferably 1 to 5. The above substitutions, deletions, insertions, or additions of one or several amino acids are conservative mutations that preserve the  $\beta$ -glucoside PTS activity. A conservative mutation is when substitution takes place mutually among Phe, Trp, Tyr, if the substitution site is an aromatic amino acid; among Leu, Ile, Val, if the substitution site is a hydrophobic amino acid; between Gln, Asn, if it is a polar amino acid; among Lys, Arg, His, if it is a basic amino acid; between Asp, Glu, if it is an acidic amino acid; and between Ser, Thr, if it is an amino acid having a hydroxyl group. Typical conservative mutations are conservative substitutions. Preferred conservative substitutions include substitution of Ala by Ser or Thr; the substitution of Arg by Gln, His, or Lys; the substitution of Asn by Glu, Gln, Lys, His, or Asp; the substitution of Asp by Asn, Glu, or Gln; the substitution of Cys by Ser or Ala; the substitution of Gln by Asn, Glu, Lys, His, Asp, or Arg; the substitution of Gly, Asn, Gln, Lys, or Asp; the substitution of Gly by Pro; the substitution of His by Asn, Lys, Gln, Arg, or Tyr; the substitution of Ile by Leu, Met, Val, or Phe; the substitution of Leu by Ile, Met, Val, or Phe; the substitution of Lys by Asn, Glu, Gln, His, or Arg; the substitution of Met by Ile, Leu, Val, or Phe; the substitution of Phe by Trp, Tyr, Met, Ile, or Leu; the substitution of Ser by Thr or Ala; the substitution of Thr by Ser or Ala; the substitution of Trp by Phe or Tyr; the substitution of Tyr by His, Phe, or Trp; and the substitution of Val by Met, Ile, or Leu. Substitutions, deletions, insertions, additions, or inversions and the like of the amino acids described above include ones that have naturally occurred (mutant or

variant) due to the differences between species, or individual differences of microorganisms that retain bglF genes. Such genes can be obtained by modifying, using, for instance, the site-specific mutation method, the nucleotide sequence shown in SEQ ID No. 5, so that the site-specific amino acid residue in the protein encoded includes substitutions, deletions, insertions, or additions.

Moreover, the bglF gene homologs can have 80% or above, preferably 90% or above, more preferably 95% or above, even more preferably 97% or above, homology with the amino acid sequence of SEQ No. 6. Since the degenerate code properties of a gene vary with the host into which the gene is introduced, a gene substituted with codons that are more readily utilized by the host is desirable. Likewise, as long as the bglF gene encodes a protein with the function of the  $\beta$ -glucoside PTS, the N terminal or C terminal of the gene may be extended or removed. For example, the number of amino acids which can be extended or removed may be 50 or less, preferably 20 or less, more preferably 10 or less, and even more preferably 5 or less. More specifically, a gene which encodes a protein with from 50 to 5 amino acids extended or removed from either end of SEQ ID No.6 may be used.

Also, a variant of the gene can be obtained by the following conventional mutation treatments. For example, a gene having a nucleotide sequence of SEQ ID No. 5 may be mutated in vitro using hydroxylamine, etc. Another method employs treating the *Escherichia* bacteria with a typical mutation treatment, such as ultraviolet light or a mutation agent, such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or Ethyl Methyl Sulfonate (EMS). Whether or not these genes encode a protein that has  $\beta$ -glucoside PTS activity can be confirmed, for example, by expressing these genes in the appropriate cells, and investigating if the ability to uptake  $\beta$ -glucoside has been increased or investigating the phosphorylating activity in vitro employing the method of Chen et al. (Biochemistry 1998 37:8714-8723).

The bglF gene can also be a DNA that hybridizes under stringent conditions with nucleotide sequences complementary to the nucleotide sequences of SEQ ID No.5, or with a probe prepared from these sequences. Here, the term "stringent conditions" refers to conditions under which so-called specific hybrids are formed and nonspecific hybrids are not formed. Although it is difficult to clearly express such conditions in numbers, these can be exemplified as conditions under which highly homologous fragments of DNA, for example, DNA having homology no less than 80%, 90%, or 95%, hybridize with each other and DNAs having homology lower than the above do not hybridize with each other. Alternatively, stringent conditions are exemplified by conditions typical of Southern hybridization washing conditions, which are to wash once or preferably two to three times at a temperature and salt concentration corresponding to 60° C., 1×SSC, 0.1% SDS, preferably 0.1×SSC, 0.1% SDS, and more preferably, 68° C., 0.1×SSC, 0.1% SDS.

DNA containing the nucleotide sequence of SEQ ID No. 5, or a part thereof may also be used as the probe. Such a probe can be prepared using PCR wherein a DNA fragment containing a nucleotide sequence of SEQ ID No. 5 is used as the template, and an oligonucleotide prepared based on the nucleotide sequence of SEQ ID No. 5 as the primer. For example, when using an approx. 300 bp long DNA fragment as the probe, the hybridization washing conditions are 50° C., 2×SSC, and 0.1% SDS.

To enhance the expression of the bglF gene, genetic recombination techniques, for example, can be employed to increase the number of copies of the above-mentioned bglF

gene in the cell. For example, a DNA fragment containing the bglF gene is ligated with a vector, preferably a multicopy type vector, which functions in the host microorganism to prepare the recombinant DNA, which is then introduced into the microorganism to transform it.

When the bglF gene of *Escherichia coli* is used, the bglF gene can be obtained by PCR (PCR: polymerase chain reaction; see White, T. J. et al., Trends Genet. 5, 185 (1989)) in which the chromosomal DNA of *Escherichia coli* is the template, and primers are prepared based on the nucleotide sequence of SEQ ID No. 5, for example, the primers shown in SEQ ID Nos. 1 and 2. The bglF genes of other microorganisms belonging to the family Enterobacteriaceae can also be obtained from the known bglF genes in those microorganisms, the bglF genes in microorganisms of other species, chromosomal DNA, or a chromosomal DNA library of microorganisms by PCR wherein the primers are prepared based on the sequence information of the BglF protein, or by hybridization wherein a probe is prepared based on the above-mentioned sequence information. Incidentally, chromosomal DNA can be prepared from DNA donor microorganisms. For example, the method of Saito and Miura, etc. (H. Saito and K. Miura, Biochem. Biophys. Acta, 72, 619 (1963), Seibutsu Kogaku Jikkensho edited by The Society of Biotechnology, Japan, pp. 97-98, Baifukan, 1992) may be used.

Next, the recombinant DNA is prepared by ligating the bglF gene(s) amplified by PCR using a vector DNA capable of functioning in the chosen host microorganism, for example, one which is autonomously replicable in the cells of the host microorganism. Examples of autonomously replicable vectors in cells of *Escherichia coli* include pUC19, pUC18, pHSG299, pHSG399, pHSG398, pACYC184, (pHSG and pACYC are available from Takara Bio Inc.), RSF1010, pBR322, pMW219 (pMW is available from Nippon Gene Co., Ltd.), pSTV29 (available from Takara Bio Inc.), etc.

Recombinant DNA prepared as described above may be introduced into a microorganism in accordance with any of the transformation methods which have been reported to date. For example, the permeability of the DNA can be increased by treating the recipient bacteria with calcium chloride, as reported with regards to *Escherichia coli* K-12 (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)). Another method is to introduce the DNA after preparing competent cells from the cells at the growth phase, as reported with regards to *Bacillus subtilis* (Duncan, C. H., Wilson, G. A. and Young, F. E., Gene, 1, 153 (1977)). Also, in relation to *Bacillus subtilis*, actinomycete and yeast, the host microorganism can be changed into the protoplast or spheroplast state that can easily uptake the recombinant DNA, which is then introduced into the DNA recipient bacteria (Chang, S. and Choen, S. N., Molec. Gen. Genet., 168, 111 (1979); Bibb, M. J., Ward, J. M. and Hopwood, O. A., Nature, 274, 398 (1978); Hinnen, A., Hicks, J. B. and Fink, G. R., Proc. Natl. Acad. Sci. USA, 75 1929 (1978)).

The copy number of the bglF gene can be increased by introducing multiple copies of the bglF gene as described above into the chromosomal DNA of the microorganism. Multiple copies of the bglF gene can be introduced into the chromosomal DNA of the microorganism by homologous recombination, using a target sequence which is present in multiple copies on the chromosomal DNA. Examples of sequences which are present in multiple copies include repetitive DNA and inverted repeats present on the ends of transposable elements. Also, these genes may be ligated in tandem with the bglF gene present on the chromosome or incorporated by duplication of unnecessary genes on the

chromosome. These genes can be introduced using a temperature-sensitive vector or integration vector.

As disclosed JP2-109985A, the bglF gene can be incorporated into a transposon, and the transposon transferred to incorporate multiple copies into the chromosomal DNA. The presence of the gene on the chromosome can be confirmed by Southern hybridization using a part of the bglF gene as a probe.

Aside from increasing the copy number described above, expression of the bglF gene can also be enhanced by employing the methods described in WO00/18935, such as substituting the expression regulatory sequence of the bglF gene promoter, etc., on the chromosomal DNA or plasmid with a stronger one, approximating the -35, -10 regions to the consensus sequence, amplifying a regulator which can enhance the expression of the bglF gene, and deleting or weakening a regulator which would decrease the expression of the bglF gene. For example, the lac promoter, trp promoter, trc promoter, tac promoter, araBA promoter, lambda phage PR promoter, PL promoter, tet promoter, T7 promoter,  $\phi$ 10 promoter, etc., are all known as strong promoters. It is also possible to introduce a base substitution, etc., into the bglF gene's promoter region and SD region to achieve greater promoter strength.

Examples of methods for evaluating the strength of promoters and examples of strong promoters are described in articles by Goldstein et al. (Prokaryotic promoters in biotechnology. Biotechnol. Annu. Rev., 1995, 1, 105-128), etc. Furthermore, the substitution of several nucleotides into the spacer region between the ribosome binding site (RBS) and the start codon, particularly into the sequence immediately upstream of the start codon, is known to have a strong effect on mRNA translation efficiency. These can be modified. The expression regulatory regions of the bglF gene's promoter, etc., can be determined by promoter search vectors and gene analysis software such as GENETYX, etc. Expression of the bglF gene can be strengthened by substitutions or modifications of these promoters. Substitution of expression regulatory sequences can be conducted, for example, by employing temperature-sensitive plasmids or the Red-driven integration method (WO2005/010175).

In order to increase the activity of a protein encoded by bglF gene, a mutation which increases the activity of a  $\beta$ -glucoside PTS may also be introduced into the bglF gene. Examples of mutations which increase the activity of the protein encoded by the bglF gene include a mutation of the promoter sequence, which increases the transcription of the bglF gene, and a mutation within the coding region of the gene, which increases the specific activity of the BglF protein.

#### <2> Method for Producing L-Amino Acid

The method for producing L-amino acids of the present invention includes culturing the microorganism of the present invention in a medium, allowing the L-amino acid to accumulate in the medium or in the microorganism, and collecting the L-amino acid from the medium or microorganism.

Media conventionally used in the fermentation of microorganism to produce L-amino acids may be used in the present invention. That is, an ordinary medium containing a carbon source, nitrogen source, non-organic ions, and other organic components as needed may be used. Carbon sources include a sugar, such as glucose, sucrose, lactose, galactose, fructose, a starch hydrolysate, etc.; an alcohol, such as glycerol, solbitol, etc.; an organic acid, such as fumaric acid, citric acid, succinic acid, etc. Of these, it is preferable to use glucose as the carbon source. Nitrogen sources include an inorganic ammonium salt, such as ammonium sulfate, ammonium chloride, ammonium phosphate, etc., an organic nitrogen, such as

a soybean hydrolysis product, etc., ammonia gas, ammonia water, etc. It is desirable for the organic micronutrient sources to contain an appropriate amount of auxotrophic substances, such as vitamin B1, L-homoserine, etc., or yeast extract, etc. In addition to these, according to necessity, small amounts of potassium phosphate, magnesium sulfate, iron ions, manganese ions, etc., can be added. The medium may be either a natural or synthetic medium as long as it contains a carbon source, nitrogen source, inorganic ions, and, as needed, other organic micronutrients.

It is recommended that the culture be performed under aerobic conditions for 1-7 days at a culture temperature of 24° C.-37° C., with a pH during the culture of 5-9. To adjust the pH, an inorganic or organic acidic or alkali substance, and ammonia gas, and the like, may be used. L-amino acids can be collected from the fermentation solution using one or a combination of a conventional methods, such as ion-exchange resin, precipitation, and other known methods. If the L-amino acid accumulates inside the cells of the microorganism, the cells can be crushed by ultrasound, etc., then removed by centrifugal separation to obtain the supernatant, from which the L-amino acid can be collected using an ion-exchange resin method, etc.

It is also possible to use a liquid medium appropriate for production of L-glutamic acid by precipitation, and to perform the culture while the L-glutamic acid is produced and collects in the medium. Conditions for production of L-glutamic acid include, for example, a pH of 5.0-4.0, preferably a pH of 4.5-4.0, more preferably a pH of 4.3-4.0, and even more preferably a pH of 4.0.

Any known recovery method may be used for collecting the L-glutamic acid from the culture solution after completion of the culture. For example, L-glutamic acid can be collected by concentration crystallization after removing the cells from the culture solution, or via ion-exchange chromatography, etc. When culturing under L-glutamic acid producing conditions, the L-glutamic acid which precipitates in the culture solution can also be collected via centrifugal separation, filtering, etc. In this case, the L-glutamic acid dissolved in the culture may be crystallized and then isolated.

Furthermore, an animal feed additive using the produced fermentation broth can be prepared by using a separation method. L-amino acid separation methods such as centrifuging, filtering, decanting, flocculating, or a combination of these can be used to remove or reduce biomass.

The obtained broth can be concentrated using known methods such as a rotary evaporator, thin layer evaporator, reverse osmosis, or nanofiltration (FR8613346B, U.S. Pat. No. 4,997,754, EP410005B, JP1073646B).

The concentrated broth is then processed using the methods of freeze-drying, spray-drying, spray granulation, or any other process to give a preferably free flowing, finely divided powder. This can then be used as an animal feed additive. This free-flowing finely divided powder can be converted into a coarse-grain, very free flowing, stable and largely dust-free product by using suitable compacting or granulating processes. Altogether, more than 90% of the water is removed in this way so that the water concentration of the animal feed additive is less than 10%, preferably less than 5% by weight.

The protein content of the feed additive can be less than 10%, preferably less than 5% by weight, and the concentration of L-threonine can be more than 50%, preferably more than 85%, more preferably more than 95% (U.S. Pat. No. 5,431,933, JP1214636B, U.S. Pat. Nos. 4,956,471, 4,777,051, 4,946,654, 5,840,358, 6,238,714, US2005/0025878).

The separation steps described above do not necessarily have to be performed, but may be combined in a technically expedient manner.

## EXAMPLES

The present invention will be explained more specifically below with reference to the following non-limiting examples.

### Reference Example 1

#### Construction of an L-Lysine-Producing Bacterium

##### <1-1> Construction of a Strain in which the *cadA* and *ldcC* Genes that Encode Lysine Decarboxylase are Disrupted

First, a strain which does not produce lysine decarboxylase was constructed. The Red-driven integration method described in WO WO2005/010175 and a  $\lambda$  phage excision system (J. Bacteriol. 2002 September; 184 (18): 5200-3. Interactions between integrase and excisionase in the phage lambda excisive nucleoprotein complex. Cho E H, Gumport R I, Gardner J F) were used to construct a strain in which lysine decarboxylase genes were disrupted. Lysine decarboxylase is encoded by the *cadA* gene (Genbank Accession No. NP\_418555. SEQ ID No. 42) and the *ldcC* gene (Genbank Accession No. NP\_414728. SEQ ID No. 44) (WO96/17930). The WC196 strain was used as the parent strain. WC196 strain was named *Escherichia coli* AJ13069, and deposited on Dec. 6, 1994 with the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology (currently, International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology; Chuo 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-8566, Japan) under Accession No. FERM P-14690 and converted to an international deposit under the Budapest Treaty on Sep. 29, 1995, and given Accession No. FERM BP-5252.

The *cadA* and *ldcC* genes encoding lysine decarboxylase were deleted using a method called "Red-driven integration," which was initially developed by Datsenko and Wanner (Proc. Natl. Acad. Sci. USA, 2000, vol. 97, No. 12, pp. 6640-6645), and a  $\lambda$  phage excision system (J. Bacteriol. 2002 September; 184 (18): 5200-3). According to the "Red-driven integration" method, it is possible to construct a gene-disrupted strain in a single step by using a PCR product obtained with a synthetic oligonucleotide primer derived from the 5' terminal end of the target gene and the 3' terminal end of the antibiotic-resistant gene. Furthermore, via  $\lambda$  phage excision, the antibiotic-resistant gene which was integrated into the chromosome can be removed from the strain.

##### (1) Disruption of the *cadA* Gene

The pMW118-attL-Cm-attR plasmid described below was used as the PCR template. pMW118-attL-Cm-attR was obtained by inserting the attL and attR-attachment site of  $\alpha$ -phage and the *cat* gene, which is an antibiotic-resistant gene, into pMW118 (Takara Bio Inc.), in the following order: attL-*cat*-attR (see WO2005/010175). The attL sequence is shown in SEQ ID No. 11, and the attR sequence is shown in SEQ ID No. 12.

PCR was conducted using the synthetic oligonucleotides shown in SEQ ID Nos. 46 and 47 as primers, wherein a sequence corresponding to both ends of attL and attR was at the primer's 3' end and a sequence corresponding to part of the *cadA* gene, the target gene, was at the primer's 5' end.

The amplified PCR product was purified with an agarose gel, then introduced by electroporation into an *Escherichia coli* WC196 strain containing plasmid pKD46, which has a

temperature-sensitive replication origin. Plasmid pKD46 (Proc. Natl. Acad. Sci. USA, 2000, vol. 97, No. 12, pp. 6640-6645) includes the  $\lambda$  phage DNA fragment (2154 bases), and the genes ( $\gamma$ ,  $\beta$ , and  $\text{exo}$ ) that encode Red recombinase in the  $\lambda$  Red homologous recombination system under the control of the arabinose-induced ParaB promoter (GenBank/EMBL Accession No. J02459, 31088th-33241st).

Competent cells for electroporation were prepared as follows. The *Escherichia coli* WC196 strain which was cultured overnight at 30° C. in an LB medium containing 100 mg/L ampicillin was diluted 100 times in a 5 mL SOB medium containing ampicillin (20 mg/L) and L-arabinose (1 mM) (Molecular Cloning: Lab Manual 2nd edition, Sambrook, J., et al., Cold Spring Harbor Laboratory Press (1989)). The dilution product was cultured at 30° C. until OD 600 grew to approx. 0.6, and then this was concentrated 100 fold and washed three times with 10% glycerol in preparation for electroporation. Electroporation was performed using 70  $\mu$ l competent cells and approx. 100 ng PCR product. 1 mL SOC medium (Molecular Cloning: Lab Manual 2<sup>nd</sup> edition, Sambrook, J., et al., Cold Spring Harbor Laboratory Press (1989)) was added and cultured at 37° C. for 2.5 hours, then cultured on a plate medium of L-agar containing Cm (chloramphenicol) (25 mg/L) at 37° C. and the Cm-resistant recombinants were selected. Next, to remove the pKD46 plasmid, cells were subcultured twice on an L-agar medium containing Cm at 42° C., the ampicillin resistance of the colony was tested, and an ampicillin-sensitive strain without pKD46 was obtained.

Deletion of the *cadA* gene in the mutant identified by the chloramphenicol-resistant gene was confirmed using PCR. The *cadA* deficient strain was designated WC196 $\Delta$ *cadA*::att-cat.

Next, to remove the att-cat gene which is introduced into the *cadA* gene, a helper plasmid, pMW-intxis-ts, described below, was used. pMW-intxis-ts contains a gene (SEQ ID No. 13) that encodes  $\lambda$  phage integrase (Int) and a gene (SEQ ID No. 15) that encodes excisionase (Xis) and has temperature-sensitive replication ability. By introducing pMW-intxis-ts, attL (SEQ ID No. 11) and attR (SEQ ID No. 12) on the chromosome are recognized, causing recombination, and the genes between attL and attR are excised, leaving only the attL or attR sequence on the chromosome.

Competent cells of the WC196 $\Delta$ *cadA*::att-cat strain obtained as described above were prepared using a typical method, and were transformed with helper plasmid pMW-intxis-ts, cultured on a plate medium of L-agar containing 50 mg/L ampicillin at 30° C., thus selecting the ampicillin-resistant strain. Next, to remove the pMW-intxis-ts plasmid, the transformants were subcultured on an L-agar medium at 42° C., the ampicillin resistance and the chloramphenicol resistance of the colony obtained were tested, and a chloramphenicol- and ampicillin-sensitive strain from which the att-cat and pMW-intxis-ts were removed was obtained. This strain was designated WC196 $\Delta$ *cadA*.

(2) Deletion of the *ldcC* Gene in the WC196 $\Delta$ *cadA* Strain

The *ldcC* gene in the WC196 $\Delta$ *cadA* strain was deleted in accordance with the technique described above, using primers having the sequences of SEQ ID Nos. 48 and 49 as the *ldcC* disrupting primers. This results in WC196 $\Delta$ *cadA* $\Delta$ *ldcC*, in which both *cadA* and *ldcC* are disrupted.

(3) Preparation of the PCR Template and Helper Plasmid

The PCR template pMW118-attL-Cm-attR and helper plasmid pMW-intxis-ts were prepared as follows.

(3-1) pMW118-attL-Cm-attR

pMW118-attL-Tc-attR was constructed based on pMW118-attL-Cm-attR. The following four DNA fragments were prepared:

1) BglIII-EcoRI DNA fragment (120 bp) (SEQ ID No. 11) containing attL obtained by PCR amplification of the sequence corresponding to the chromosome of the *E. coli* W3350 strain (ATCC31278 containing  $\lambda$  prophage), using oligonucleotides P1 and P2 (SEQ ID Nos. 17 & 18) as primers (these primers additionally contained the recognition sites of the BglIII and EcoRI endonucleases),

2) PstI-HindIII DNA fragment (182 bp) (SEQ ID No. 12) containing attR obtained by PCR amplification of the sequence corresponding to the chromosome of the *E. coli* W3350 strain (containing  $\lambda$  prophage), using oligonucleotides P3 and P4 (SEQ ID Nos. 19 & 20) as primers (these primers additionally contained the recognition sites of the PstI and HindIII endonucleases),

3) BglIII-HindIII large fragment (3916 bp) of pMW118-ter\_rnB: The pMW118-ter\_rnB was obtained by ligating the following three fragments:

i) A large fragment (2359 bp) containing an AatII-EcoRI-pol fragment from pMW118 obtained by digesting the pMW118 with an EcoRI restriction endonuclease, treating it with a Klenow fragment of DNA polymerase I, then digesting the fragment with an AatII restriction endonuclease,

ii) An AatII-BglIII small fragment (1194 bp) of pUC19 containing the ampicillin-resistant ( $\text{Ap}^R$ ) *bla* gene obtained by PCR-amplifying the sequence corresponding to the pUC19 plasmid, using oligonucleotides P5 and P6 (SEQ ID Nos. 21 & 22) as primers (these primers additionally contained the recognition sites of the AatII and BglIII endonucleases),

iii) A small BglIII-PstI-pol fragment (363 bp) containing transcription terminator *ter\_rnB* obtained by PCR-amplifying the region corresponding to the chromosome of the *E. coli* MG1655 strain, using oligonucleotides P7 and P8 (SEQ ID Nos. 23 & 24) as primers (these primers additionally contained the recognition sites of the BglIII and PstI endonucleases),

4) A small EcoRI-PstI fragment (1388 bp) (SEQ ID No. 29) of pML-Tc-ter\_thrL containing a tetracycline-resistant gene and transcription terminator *ter\_thrL*. The pML-Tc-ter\_thrL was obtained as follows.

A pML-MSC (Mol Biol (Mosk). 2005 September-October; 39(5):823-31; Biotechnologiya (Russian) No. 5: 3-20.) was digested with XbaI and BamHI restriction endonucleases, and a large fragment of this (3342 bp) was ligated with an XbaI-BamHI fragment (68 bp) that contained the terminator *ter\_thrL*. The XbaI-BamHI fragment (68 bp) corresponded to the chromosome of *E. coli* MG1655, and was obtained by PCR amplification, using oligonucleotides P9 and P10 (SEQ ID Nos. 25 & 26) as primers (these primers additionally contained the recognition sites of the XbaI and BamHI endonucleases). The ligated reaction product was designated plasmid pML-ter\_thrL.

The pML-ter\_thrL was digested with KpnI and XbaI restriction endonucleases, treated with a Klenow fragment of DNA polymerase I, then ligated with a small EcoRI-Van911 fragment (1317 bp) of pBR322 containing the tetracycline-resistant gene (the pBR322 which was digested with EcoRI and Van911 restriction endonucleases was treated with a Klenow fragment of DNA polymerase I). The product of this ligation was designated plasmid pML-Tc-ter\_thrL.

Next, the pMW118-attL-Cm-attR was constructed by ligation of a large BamHI-XbaI fragment (4413 bp), a PA2 promoter (initial promoter of T7 phage), a chloramphenicol-resistant (*CmR*) *cat* gene, an artificial BglIII-XbaI DNA fragment (1162 bp) containing transcription terminator *ter\_thrL*, and attR. The artificial DNA fragment (SEQ ID No. 30) was obtained as follows.

pML-MSC (Mol Biol (Mosk). 2005 September-October; 39(5):823-31; Biotechnologiya (Russian) No. 5: 3-20.) was digested with KpnI and XbaI restriction endonucleases, and ligated with a small KpnI-XbaI fragment (120 bp) containing a PA2 promoter (early promoter of T7 phage). A KpnI-XbaI fragment was obtained by amplifying the region corresponding to T7 phage DNA, using oligonucleotides P11 and P12 (SEQ ID Nos. 27 & 28) as primers (these primers additionally contained the recognition sites of the KpnI and XbaI endonucleases) by PCR. The product of the ligation was designated plasmid pML-PA2-MCS.

The XbaI site was removed from pML-PA2-MCS. The product was designated plasmid pML-PA2-MCS(XbaI-).

A small BglIII-HindIII fragment (928 bp) of pML-PA2-MCS(XbaI-) containing a PA2 promoter (initial promoter of T7 phage) and chloramphenicol-resistant (CrnR) cat gene was ligated with a small HindIII-HindIII fragment (234 bp) of pMW118-attL-Tc-attR, which contained the transcription terminator *ter\_thrL*, and *attR*.

The target artificial DNA fragment (1156 bp) was obtained by PCR amplification of the ligation mixture, using oligonucleotides P9 and P4 (SEQ ID Nos. 25 & 20) as primers (these primers contained the recognition sites of the HindIII and XbaI endonucleases).

#### (3-2) pMW-intxis-ts

First, two DNA fragments were amplified based on  $\lambda$  phage DNA (Fermentas) as the template. The first fragment consisted of a region of nt 37168-38046 of the genome of  $\lambda$  phage DNA (SEQ ID No. 39), and contained a *cI* repressor, *Prm* and *Pr* promoters, and the leader sequence of the *cro* gene. This fragment was obtained by amplification, using oligonucleotides P1' and P2' (SEQ ID Nos. 31 & 32) as primers. The second fragment consisted of a region of nt 27801-29100 of the genome of K phage DNA (SEQ ID No. 40), which contained the *xis-int* gene from K phage DNA. This fragment was obtained by PCR, using oligonucleotides P3' and P4' (SEQ ID Nos. 33 & 34) as primers. All of the primers contained the proper endonuclease recognition sites.

The first PCR-amplified fragment, which contained the *cI* repressor, was digested with a *Cla*I restriction endonuclease, and then digested with *Eco*RI restriction endonuclease.

The second PCR fragment was digested with *Eco*RI and *Pst*I endonucleases. The plasmid pMWPlaclacI-ts was digested with *Bgl*III endonuclease, treated with a Klenow fragment of DNA polymerase I, and then digested with a *Pst*I restriction endonuclease. A vector fragment of pMWPlaclacI-ts was eluted from an agarose gel and ligated with the cut PCR-amplified fragment.

The plasmid pMWPlaclacI-ts is a derivative of pMWPlaclacI containing the following parts: 1) an artificial *Bgl*III-HindIII DNA fragment containing a *PlacUV5* promoter and the *lacI* gene under control of the RBS of the bacteriophage T7 gene 10; 2) an *Aat*II-*Bgl*III fragment containing the ampicillin-resistant (*Ap<sup>R</sup>*) gene obtained by PCR amplification of the region corresponding to the pUC19 plasmid, using oligonucleotides P5' and P6' (SEQ ID Nos. 35 & 36) as primers (these primers contained the recognition sites of the *Aat*II and *Bgl*III endonucleases); 3) an *Aat*II-HindIII fragment containing an *Aat*II-*Pvu*I fragment of a recombinant plasmid pMW118-*ter\_rrnB*. The plasmid pMW118-*ter\_rrnB* was constructed as follows. A *Pst*I-HindIII fragment containing a terminator *ter\_rrnB* was obtained by PCR amplification of the region corresponding to the chromosome of the *E. coli* MG1655 strain, using as primers oligonucleotides P7' and P8' (SEQ ID Nos. 37 & 38) which contained the proper endonuclease recognition sites. Prior to ligation, the pMW118 and *ter\_rrnB* fragments (complementary strand of SEQ ID No.

41) were digested with *Pvu*I or *Pst*I, respectively, treated with a Klenow fragment of DNA polymerase I to blunt the ends, and then digested with *Aat*II or *Hind*III endonuclease. In the construction of the pMWPlaclacI-ts mutant, an *Aat*II-*Eco*RV fragment of plasmid pMWPlaclacI was substituted with an *Aat*II-*Eco*RV fragment of plasmid pMAN997 which contained the *par*, *ori*, and *repA*ts genes of the pSC101 replicon. (Applied and Environmental Microbiology, June 2005, p. 3228-32)

#### Example 1

##### Construction of Plasmid for *bglF* Overexpression

The total genome sequence of the chromosome of *Escherichia coli* (*Escherichia coli* K-12 strain) has been determined (Science, 277, 1453-1474 (1997)). Based on the nucleotide sequence of the *bglF* gene, using as a 5' primer the synthetic oligonucleotide of SEQ ID No.1 having a *Hind*III site, and as a 3' primer the synthetic oligonucleotide of SEQ ID No.2 having an *Xba*I site, PCR was performed using chromosomal DNA of the *Escherichia Coli* MG1655 strain as the template. The PCR product was treated with restriction endonucleases *Hind*III and *Xba*I, and a gene fragment that contained the *bglF* genes was obtained.

The purified PCR product was ligated with vector pMW219 which had been digested with *Hind*III and *Xba*I (Nippon Gene Co., Ltd.) to construct a plasmid pM-*bglF* for *bglF* overexpression. This plasmid was under the control of a *lac* promoter and the *bglF* gene was placed downstream of the *lac* promoter. pM-*bglF* was digested with *Hind*III and *Eco*RI, the *bglF* gene fragments were collected and purified, and ligated to vector pSTV29 which had been digested with *Hind*III and *Eco*RI (Takara Shuzo). In this way, the plasmid pS-*bglF* for *bglF* overexpression was constructed.

In the same manner as with the above-mentioned *bglF* gene, a plasmid for expressing the *ptsG* gene was constructed as the control. The sequence of *ptsG* is shown in SEQ ID No. 7 and the sequence of the amino acid is given in SEQ ID No. 8; the *ptsG* sequence can be obtained with reference to Genbank Accession No. NP\_415619. Using as a 5' primer the synthetic oligonucleotide of SEQ ID No.3 containing a *Hind*III site, and as a 3' primer the synthetic oligonucleotide of SEQ ID No.4 containing an *Xba*I site, PCR was performed using the chromosomal DNA of the *Escherichia Coli* MG1655 strain as the template, and the PCR fragment was treated with restriction endonucleases *Hind*III and *Xba*I, and a gene fragment containing *ptsG* was obtained. The purified PCR product was ligated with vector pMW219, which had been digested with *Hind*III and *Xba*I, to construct plasmid pM-*ptsG* for *ptsG* overexpression. This plasmid was under the control of a *lac* promoter, and the *ptsG* gene was placed downstream of the *lac* promoter. In the same manner as with the *bglF*, the *ptsG* gene fragment was excised from the pM-*ptsG*, and ligated to vector pSTV29. In this way, the plasmid pS-*ptsG* for *ptsG* overexpression was constructed.

#### Example 2

##### Construction of the Strain in which the *bglF* Gene is Overexpressed and Evaluation of L-Lysine Production of the Strain

As an *Escherichia coli* L-lysine-producing strain, the WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2) strain was used as parent strain. Lys-producing plasmid pCABD2 carrying the *dapA*, *dapB*, and *lysC* genes (WO01/53459) was introduced into the



WC196 $\Delta$ ldcC $\Delta$ cadA strain. The WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2) strain was transformed with the bglF-overexpression plasmid pM-bglF and the ptsG-overexpression plasmid pM-ptsG constructed in Example 1, and the control plasmid pMW219, and kanamycin-resistant strains were obtained. After confirming that these plasmids had been introduced, the bglF-overexpression plasmid pM-bglF-introduced strain was designated WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2, pM-bglF); the ptsG-overexpression plasmid pM-ptsG-introduced strain was designated WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2, pM-ptsG); and the control plasmid pMW219-introduced strain was designated WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2, pMW219).

The strains constructed as described above were cultured in an L medium containing 25 mg/L kanamycin at 37° C. to finally become OD600 0.6. Then, an equal volume of a 40% glycerol solution was added to the culture and stirred, then appropriate amounts were pipetted and stored at -80° C. This was called the glycerol stock.

After melting the glycerol stock of these strains, 100  $\mu$ L of each was evenly spread onto an L plate containing 25 mg/L kanamycin, and this was cultured at 37° C. for 24 hours. Approx. 1/8 of the cells on the plate were inoculated into a 20 mL fermentation medium (shown below) with 25 mg/L kanamycin in a 500 mL Sakaguchi shaking flask, and cultured at 37° C. for 24 hours using a reciprocating shaking culture apparatus. After culturing, the amount of lysine which had accumulated in the medium was measured using a Biotech-analyzer AS210 (Sakura Seiki).

The OD and L-lysine which had accumulated at the 24th hour are shown in Table 1. As evident in Table 1, a large amount of L-lysine accumulated in the WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2, pM-bglF) strain, compared to the WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2, pMW219) strain which did not contain the bglF genes. An improvement in the amount of L-lysine which accumulated was also confirmed in comparison with the WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2, pM-ptsG) strain, which did contain the ptsG gene. Such data shows that overexpression of the bglF gene is more effective in lysine production than overexpression of the ptsG.

TABLE 1

Bacterial strain	OD600	Lys-HCl(g/L)
WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2, pMW219)	12.6	10.0
WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2, pM-bglF)	17.1	16.1
WC196 $\Delta$ ldcC $\Delta$ cadA (pCABD2, pM-ptsG)	15.8	14.7

#### Medium for L-Lysine Production:

Glucose	40 g/L
Ammonium sulfate	24 g/L
Potassium Dihydrogen Phosphate	1.0 g/L
Magnesium sulfate 7-hydrate	1.0 g/L
Ferrous sulfate 4•7-hydrate	0.01 g/L
Manganese sulfate 4•7-hydrate	0.01 g/L
Yeast extract	2.0 g/L
Calcium carbonate	30 g/L

Adjusted to pH 7.0 with KOH, and sterilized at 115° C. for 10 min.  
Glucose and MgSO<sub>4</sub>•7H<sub>2</sub>O were sterilized separately.

### Example 3

#### Effect of bglF Overexpression on an L-Glutamic Acid-Producing Strain of *Escherichia* Bacteria

As an *Escherichia coli* L-glutamic acid-producing strain, the AJ12949 strain was used as the parent strain. The AJ12949

strain is a bacterial strain in which the  $\alpha$ -ketoglutarate dehydrogenase activity has been reduced, and was deposited on Dec. 28, 1993 with the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology (currently, International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology; Chuo 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-8566, Japan) under Accession No. FERM P-14039 and converted to an international deposit under the Budapest Treaty on Nov. 11, 1994, and given Accession No. FERM BP-4881.

The AJ12949 strain was transformed with the bglF-overexpression plasmid pS-bglF used in Example 1, and the control plasmid pSTV29, and chloramphenicol-resistant strains were obtained. After confirming that the plasmids had been introduced, the strain into which the bglF-overexpression plasmid pS-bglF was introduced was designated AJ12949 (pS-bglF); and the strain into which the control plasmid pSTV29 was introduced was designated AJ12949 (pSTV29).

The AJ12949 (pS-bglF) strain and the AJ12949 (pSTV29) strain were cultured in an L medium containing 20 mg/L chloramphenicol at 37° C. to finally become OD600 0.6. After this, an equal volume of a 40% glycerol solution was added to the culture and stirred, then appropriate amounts were pipetted to obtain a glycerol stock and stored at -80° C.

After melting the glycerol stock of these strains, 100  $\mu$ L of each was evenly spread onto an L plate containing 20 mg/L chloramphenicol, and cultured at 37° C. for 24 hours. Approx. 1/8 of the cells on the plate were inoculated into a 20 mL fermentation medium (described below) with 20 mg/L chloramphenicol in a 500 mL Sakaguchi flask, and cultured at 37° C. for 40 hours using a reciprocating shaking culture apparatus. After culturing, the amount of L-glutamic acid which had accumulated in the medium was measured using a Biotech-analyzer AS210 (Sakura Seiki).

The OD and L-glutamic acid which had accumulated at the 40<sup>th</sup> hour are shown in Table 2. As shown in Table 2, a large amount of L-glutamic acid had accumulated in the AJ12949 (pS-bglF) strain, compared to the AJ12949 (pSTV29) strain which did not contain the bglF genes.

TABLE 2

Bacterial strain	OD600	L-Glu (g/L)
AJ12949 (pSTV29)	14.7	18.6
AJ12949 (pS- bglF)	16.6	20.4

#### Medium for L-Glutamic Acid-Production:

Glucose	40 g/L
Ammonium sulfate	20 g/L
Potassium Dihydrogen Phosphate	1.0 g/L
Magnesium sulfate 7-hydrate	1.0 g/L
Ferrous sulfate 4•7-hydrate	0.01 g/L
Manganese sulfate 4•7-hydrate	0.01 g/L
Yeast extract	2.0 g/L
Calcium carbonate	30 g/L

Adjusted to pH 7.0 with KOH, sterilized at 115° C. for 10 min.

Glucose and MgSO<sub>4</sub>•7H<sub>2</sub>O were sterilized separately.

Also, after the culture temperature was at 60° C. or below, a thiamine hydrochloride solution which had been sterilized with a DISMIC-25cs 0.2 mm filter (ADVANTEC) was added to obtain the final concentration of 0.01 g/L.

Effect of bglF Overexpression on an  
L-Threonine-Producing Strain of Bacteria of the  
Genus *Escherichia*

As the parent strain of the bglF overexpression for L-threonine-production, the B-5318 strain was used. The B-5318 strain was deposited on May 3, 1990 with the Russian National Collection of Industrial Microorganisms (VKPM), GNII Genetika (Russia, 117545 Moscow, 1 Dorozhny Proezd, 1) under Accession No. VKPM B-5318. The construction of the bglF overexpression strain from B-5318 was performed using the plasmid as described in Example 1.

The B-5318 strain was transformed with the bglF-amplifying plasmid pS-bglF used in Example 1 and the control plasmid pSTV29, and chloramphenicol-resistant strains were obtained. After confirming that the prescribed plasmids had been introduced, the strain into which bglF-overexpression plasmid pS-bglF was introduced was designated B-5318 (pS-bglF); and the strain into which control plasmid pSTV29 was introduced was designated B-5318 (pSTV29).

The B-5318 (pS-bglF) strain and the B-5318 (pSTV29) strain were cultured in an L medium containing 20 mg/L chloramphenicol at 37° C. to finally become OD600 0.6. After this, an equal volume of a 40% glycerol solution was added to the culture and stirred, then appropriate amounts were pipetted to obtain a glycerol stock and stored at -80° C.

After melting the glycerol stock of these strains, 100 µL of each was evenly spread onto an L plate containing 20 mg/L chloramphenicol, and cultured at 37° C. for 24 hours. Approx. 1/8 of the cells on the plate were inoculated into a 20 mL fermentation medium with 20 mg/L chloramphenicol in a 500 mL Sakaguchi shaking flask, and cultured at 37° C. for 16 hours using a reciprocating shaking culture apparatus. After culturing, the amount of L-threonine which had accumulated in the medium was measured using high-performance liquid chromatography.

The OD and L-threonine which had accumulated at the 16th hour are shown in Table 3. As shown in the table, a large amount of L-threonine had accumulated in the B-5318 (pS-bglF) strain, compared to the B-5318 (pSTV29) strain, which did not contain the bglF gene.

TABLE 3

Bacterial strain	OD600	L-threonine (g/L)
B-5318 (pSTV29)	8.0	3.6
B-5318 (pS-bglF)	10.3	4.4

5	Glucose	60 g/L
	Ammonium sulfate	16 g/L
	Potassium Dihydrogen Phosphate	0.7 g/L
	Magnesium sulfate 7-hydrate	1.0 g/L
	Ferrous sulfate 7-hydrate	0.01 g/L
	Manganese sulfate 7-hydrate	0.01 g/L
10	Yeast extract	0.5 g/L
	Thiamine hydrochloride	0.2 mg/L
	L-isoleucine	0.05 g/L
	Calcium carbonate	30 g/L

Adjusted to pH 7.0 with KOH, sterilized at 115° C. for 10 min.

15 However, glucose and MgSO<sub>4</sub>·7H<sub>2</sub>O were sterilized separately. Potassium hydroxide was sterilized by dry heat at 180° C. for 3 hours. After the culture temperature came down to 60° C. or lower, a thiamine hydrochloride solution which had been sterilized with a DISMIC-25cs 0.2 mm filter (ADVANTEC) was added to obtain the final concentration of 0.2 mg/L.

## Example 5

Effect of bglF Overexpression on an L-Glutamic Acid-Producing Strain of *Pantoea* Bacteria

25 As the parent strain of the bglF amplification L-glutamic acid-producing strain, the *Pantoea ananatis* AJ13601 strain can be used. The *Pantoea ananatis* AJ13601 strain was deposited on Aug. 18, 1999 with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of Economy, Trade and Industry (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-8566) under Accession No. FERM P-17516 and converted to an international deposit under the Budapest Treaty on Jul. 6, 2000, and given Accession No. FERM BP-7207. 30 The bglF amplified strains can be constructed from L-glutamic acid-producing bacteria using the plasmid described in Example 1.

35 The bglF overexpressed strains are cultured in an L-glutamic acid-production medium and cultured using a reciprocating shaking culture apparatus. After culturing, the amount of L-glutamic acid which had accumulated in the medium is measured using Biotech-analyzer AS210 (Sakura Seiki) to confirm whether the accumulation of L-glutamic acid has increased. In this way, the bglF overexpressed strain with an improved L-glutamic acid-producing ability can be 40 obtained.

45 While the invention has been described in detail with reference to preferred embodiments thereof, it will be apparent to one skilled in the art that various changes can be made, and equivalents employed, without departing from the scope of the invention. All documents cited herein are hereby incorporated by reference. 50

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tac acc aac gca gca ggt cag gtt ttc cac ggc gac att ccg cgt tat Tyr Thr Asn Ala Ala Gly Gln Val Phe His Gly Asp Ile Pro Arg Tyr 225 230 235 240	720
atg gcg ggt gac ccg act gcg ggt aaa ctg tct ggt ggc ttc ctg ttc Met Ala Gly Asp Pro Thr Ala Gly Lys Leu Ser Gly Gly Phe Leu Phe 245 250 255	768
aaa atg tac ggt ctg cca gct gcc gca att gct atc tgg cac tct gct Lys Met Tyr Gly Leu Pro Ala Ala Ile Ala Ile Trp His Ser Ala 260 265 270	816
aaa cca gaa aac cgc gcg aaa gtg ggc ggt att atg atc tcc gcg gcg Lys Pro Glu Asn Arg Ala Lys Val Gly Gly Ile Met Ile Ser Ala Ala 275 280 285	864
ctg acc tcg ttc ctg acc ggt atc acc gag ccg atc gag ttc tcc ttc Leu Thr Ser Phe Leu Thr Gly Ile Thr Glu Pro Ile Glu Phe Ser Phe 290 295 300	912
atg ttc gtt gcg ccg atc ctg tac atc atc cac gcg att ctg gca ggc Met Phe Val Ala Pro Ile Leu Tyr Ile Ile His Ala Ile Leu Ala Gly 305 310 315 320	960
ctg gca ttc cca atc tgt att ctt ctg ggg atg cgt gac ggt acg tcg Leu Ala Phe Pro Ile Cys Ile Leu Leu Gly Met Arg Asp Gly Thr Ser 325 330 335	1008
ttc tcg cac ggt ctg atc gac ttc atc gtt ctg tct ggt aac agc agc Phe Ser His Gly Leu Ile Asp Phe Ile Val Leu Ser Gly Asn Ser Ser 340 345 350	1056
aaa ctg tgg ctg ttc ccg atc gtc ggt atc ggt tat gcg att gtt tac Lys Leu Trp Leu Phe Pro Ile Val Gly Ile Gly Tyr Ala Ile Val Tyr 355 360 365	1104
tac acc atc ttc cgc gtg ctg att aaa gca ctg gat ctg aaa acg ccg Tyr Thr Ile Phe Arg Val Leu Ile Lys Ala Leu Asp Leu Lys Thr Pro 370 375 380	1152
ggt cgt gaa gac gcg act gaa gat gca aaa gcg aca ggt acc agc gaa Gly Arg Glu Asp Ala Thr Glu Asp Ala Lys Ala Thr Gly Thr Ser Glu 385 390 395 400	1200
atg gca ccg gct ctg gtt gct gca ttt ggt ggt aaa gaa aac att act Met Ala Pro Ala Leu Val Ala Ala Phe Gly Gly Lys Glu Asn Ile Thr 405 410 415	1248
aac ctc gac gca tgt att acc cgt ctg cgc gtc agc gtt gct gat gtg Asn Leu Asp Ala Cys Ile Thr Arg Leu Arg Val Ser Val Ala Asp Val 420 425 430	1296
tct aaa gtg gat cag gcc ggc ctg aag aaa ctg ggc gca gcg ggc gta Ser Lys Val Asp Gln Ala Gly Leu Lys Lys Leu Gly Ala Ala Gly Val 435 440 445	1344
gtg gtt gct ggt tct ggt gtt cag gcg att ttc ggt act aaa tcc gat Val Val Ala Gly Ser Gly Val Gln Ala Ile Phe Gly Thr Lys Ser Asp 450 455 460	1392
aac ctg aaa acc gag atg gat gag tac atc cgt aac cac taa Asn Leu Lys Thr Glu Met Asp Glu Tyr Ile Arg Asn His 465 470 475	1434

&lt;210&gt; SEQ ID NO 8

&lt;211&gt; LENGTH: 477

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Escherichia coli



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&lt;400&gt; SEQUENCE: 8

Met Phe Lys Asn Ala Phe Ala Asn Leu Gln Lys Val Gly Lys Ser Leu  
 1 5 10 15  
 Met Leu Pro Val Ser Val Leu Pro Ile Ala Gly Ile Leu Leu Gly Val  
 20 25 30  
 Gly Ser Ala Asn Phe Ser Trp Leu Pro Ala Val Val Ser His Val Met  
 35 40 45  
 Ala Glu Ala Gly Gly Ser Val Phe Ala Asn Met Pro Leu Ile Phe Ala  
 50 55 60  
 Ile Gly Val Ala Leu Gly Phe Thr Asn Asn Asp Gly Val Ser Ala Leu  
 65 70 75 80  
 Ala Ala Val Val Ala Tyr Gly Ile Met Val Lys Thr Met Ala Val Val  
 85 90 95  
 Ala Pro Leu Val Leu His Leu Pro Ala Glu Glu Ile Ala Ser Lys His  
 100 105 110  
 Leu Ala Asp Thr Gly Val Leu Gly Gly Ile Ile Ser Gly Ala Ile Ala  
 115 120 125  
 Ala Tyr Met Phe Asn Arg Phe Tyr Arg Ile Lys Leu Pro Glu Tyr Leu  
 130 135 140  
 Gly Phe Phe Ala Gly Lys Arg Phe Val Pro Ile Ile Ser Gly Leu Ala  
 145 150 155 160  
 Ala Ile Phe Thr Gly Val Val Leu Ser Phe Ile Trp Pro Pro Ile Gly  
 165 170 175  
 Ser Ala Ile Gln Thr Phe Ser Gln Trp Ala Ala Tyr Gln Asn Pro Val  
 180 185 190  
 Val Ala Phe Gly Ile Tyr Gly Phe Ile Glu Arg Cys Leu Val Pro Phe  
 195 200 205  
 Gly Leu His His Ile Trp Asn Val Pro Phe Gln Met Gln Ile Gly Glu  
 210 215 220  
 Tyr Thr Asn Ala Ala Gly Gln Val Phe His Gly Asp Ile Pro Arg Tyr  
 225 230 235 240  
 Met Ala Gly Asp Pro Thr Ala Gly Lys Leu Ser Gly Gly Phe Leu Phe  
 245 250 255  
 Lys Met Tyr Gly Leu Pro Ala Ala Ala Ile Ala Ile Trp His Ser Ala  
 260 265 270  
 Lys Pro Glu Asn Arg Ala Lys Val Gly Gly Ile Met Ile Ser Ala Ala  
 275 280 285  
 Leu Thr Ser Phe Leu Thr Gly Ile Thr Glu Pro Ile Glu Phe Ser Phe  
 290 295 300  
 Met Phe Val Ala Pro Ile Leu Tyr Ile Ile His Ala Ile Leu Ala Gly  
 305 310 315 320  
 Leu Ala Phe Pro Ile Cys Ile Leu Leu Gly Met Arg Asp Gly Thr Ser  
 325 330 335  
 Phe Ser His Gly Leu Ile Asp Phe Ile Val Leu Ser Gly Asn Ser Ser  
 340 345 350  
 Lys Leu Trp Leu Phe Pro Ile Val Gly Ile Gly Tyr Ala Ile Val Tyr  
 355 360 365  
 Tyr Thr Ile Phe Arg Val Leu Ile Lys Ala Leu Asp Leu Lys Thr Pro  
 370 375 380  
 Gly Arg Glu Asp Ala Thr Glu Asp Ala Lys Ala Thr Gly Thr Ser Glu  
 385 390 395 400  
 Met Ala Pro Ala Leu Val Ala Ala Phe Gly Gly Lys Glu Asn Ile Thr  
 405 410 415

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Asn Leu Asp Ala Cys Ile Thr Arg Leu Arg Val Ser Val Ala Asp Val  
                   420                                  425                                  430

Ser Lys Val Asp Gln Ala Gly Leu Lys Lys Leu Gly Ala Ala Gly Val  
                   435                                  440                                  445

Val Val Ala Gly Ser Gly Val Gln Ala Ile Phe Gly Thr Lys Ser Asp  
                   450                                  455                                  460

Asn Leu Lys Thr Glu Met Asp Glu Tyr Ile Arg Asn His  
 465                                  470                                  475

<210> SEQ ID NO 9  
 <211> LENGTH: 101  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: lambda phage

<400> SEQUENCE: 9

cctgcttttt tataactaagt tggcattata aaaaagcatt gcttatcaat ttgttgcaac 60  
 gaacaggtca ctatcagtc aaataaaatc attatttgat t 101

<210> SEQ ID NO 10  
 <211> LENGTH: 172  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: lambda phage

<400> SEQUENCE: 10

gcgctaagtc tctgttacag gtcactaata ccatactaat agttgattca tagtgactgc 60  
 atatgttggtg ttttacagta ttatgtagtc tgttttttat gcaaaatcta atttaataata 120  
 ttgatattta taccatttta cgtttctcgt tcagcttttt tataactaact tg 172

<210> SEQ ID NO 11  
 <211> LENGTH: 120  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: lambda phage

<400> SEQUENCE: 11

agatcttgaa gcctgctttt ttataactaag ttggcattat aaaaaagcat tgcttatcaa 60  
 tttgttgcaa cgaacaggtc actatcagtc aaaataaaat cattatttga tttcgaattc 120

<210> SEQ ID NO 12  
 <211> LENGTH: 184  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: lambda phage

<400> SEQUENCE: 12

ctgcagtcctg ttacaggtca ctaataccat ctaagtagtt gattcatagt gactgcatat 60  
 gttgtgtttt acagtattat gtagtctggt ttttatgcaa aatctaattt aatatattga 120  
 tatttatatc attttacgtt tctcgttcag cttttttata ctaacttgag cgtctagaaa 180  
 gctt 184

<210> SEQ ID NO 13  
 <211> LENGTH: 1071  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence



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ttt atg cgc gca cga aaa gca tca ggt ctt tcc ttc gaa ggg gat ccg	912
Phe Met Arg Ala Arg Lys Ala Ser Gly Leu Ser Phe Glu Gly Asp Pro	
290 295 300	
cct acc ttt cac gag ttg cgc agt ttg tct gca aga ctc tat gag aag	960
Pro Thr Phe His Glu Leu Arg Ser Leu Ser Ala Arg Leu Tyr Glu Lys	
305 310 315 320	
cag ata agc gat aag ttt gct caa cat ctt ctc ggg cat aag tcg gac	1008
Gln Ile Ser Asp Lys Phe Ala Gln His Leu Leu Gly His Lys Ser Asp	
325 330 335	
acc atg gca tca cag tat cgt gat gac aga ggc agg gag tgg gac aaa	1056
Thr Met Ala Ser Gln Tyr Arg Asp Asp Arg Gly Arg Glu Trp Asp Lys	
340 345 350	
att gaa atc aaa taa	1071
Ile Glu Ile Lys	
355	

<210> SEQ ID NO 14  
 <211> LENGTH: 356  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: lambda phage

<400> SEQUENCE: 14

Met Gly Arg Arg Arg Ser His Glu Arg Arg Asp Leu Pro Pro Asn Leu	
1 5 10 15	
Tyr Ile Arg Asn Asn Gly Tyr Tyr Cys Tyr Arg Asp Pro Arg Thr Gly	
20 25 30	
Lys Glu Phe Gly Leu Gly Arg Asp Arg Arg Ile Ala Ile Thr Glu Ala	
35 40 45	
Ile Gln Ala Asn Ile Glu Leu Phe Ser Gly His Lys His Lys Pro Leu	
50 55 60	
Thr Ala Arg Ile Asn Ser Asp Asn Ser Val Thr Leu His Ser Trp Leu	
65 70 75 80	
Asp Arg Tyr Glu Lys Ile Leu Ala Ser Arg Gly Ile Lys Gln Lys Thr	
85 90 95	
Leu Ile Asn Tyr Met Ser Lys Ile Lys Ala Ile Arg Arg Gly Leu Pro	
100 105 110	
Asp Ala Pro Leu Glu Asp Ile Thr Thr Lys Glu Ile Ala Ala Met Leu	
115 120 125	
Asn Gly Tyr Ile Asp Glu Gly Lys Ala Ala Ser Ala Lys Leu Ile Arg	
130 135 140	
Ser Thr Leu Ser Asp Ala Phe Arg Glu Ala Ile Ala Glu Gly His Ile	
145 150 155 160	
Thr Thr Asn His Val Ala Ala Thr Arg Ala Ala Lys Ser Glu Val Arg	
165 170 175	
Arg Ser Arg Leu Thr Ala Asp Glu Tyr Leu Lys Ile Tyr Gln Ala Ala	
180 185 190	
Glu Ser Ser Pro Cys Trp Leu Arg Leu Ala Met Glu Leu Ala Val Val	
195 200 205	
Thr Gly Gln Arg Val Gly Asp Leu Cys Glu Met Lys Trp Ser Asp Ile	
210 215 220	
Val Asp Gly Tyr Leu Tyr Val Glu Gln Ser Lys Thr Gly Val Lys Ile	
225 230 235 240	
Ala Ile Pro Thr Ala Leu His Ile Asp Ala Leu Gly Ile Ser Met Lys	
245 250 255	
Glu Thr Leu Asp Lys Cys Lys Glu Ile Leu Gly Gly Glu Thr Ile Ile	
260 265 270	

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Ala Ser Thr Arg Arg Glu Pro Leu Ser Ser Gly Thr Val Ser Arg Tyr  
 275 280 285

Phe Met Arg Ala Arg Lys Ala Ser Gly Leu Ser Phe Glu Gly Asp Pro  
 290 295 300

Pro Thr Phe His Glu Leu Arg Ser Leu Ser Ala Arg Leu Tyr Glu Lys  
 305 310 315 320

Gln Ile Ser Asp Lys Phe Ala Gln His Leu Leu Gly His Lys Ser Asp  
 325 330 335

Thr Met Ala Ser Gln Tyr Arg Asp Asp Arg Gly Arg Glu Trp Asp Lys  
 340 345 350

Ile Glu Ile Lys  
 355

<210> SEQ ID NO 15  
 <211> LENGTH: 219  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <221> NAME/KEY: CDS  
 <222> LOCATION: (1)..(219)  
 <223> OTHER INFORMATION: lambda phage

<400> SEQUENCE: 15

atg tac ttg aca ctt cag gag tgg aac gca cgc cag cga cgt cca aga 48  
 Met Tyr Leu Thr Leu Gln Glu Trp Asn Ala Arg Gln Arg Arg Pro Arg  
 1 5 10 15

agc ctt gaa aca gtt cgt cga tgg gtt cgg gaa tgc agg ata ttc cca 96  
 Ser Leu Glu Thr Val Arg Arg Trp Val Arg Glu Cys Arg Ile Phe Pro  
 20 25 30

cct ccg gtt aag gat gga aga gag tat ctg ttc cac gaa tca gcg gta 144  
 Pro Pro Val Lys Asp Gly Arg Glu Tyr Leu Phe His Glu Ser Ala Val  
 35 40 45

aag gtt gac tta aat cga cca gta aca ggt ggc ctt ttg aag agg atc 192  
 Lys Val Asp Leu Asn Arg Pro Val Thr Gly Gly Leu Leu Lys Arg Ile  
 50 55 60

aga aat ggg aag aag gcg aag tca tga 219  
 Arg Asn Gly Lys Lys Ala Lys Ser  
 65 70

<210> SEQ ID NO 16  
 <211> LENGTH: 72  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: lambda phage

<400> SEQUENCE: 16

Met Tyr Leu Thr Leu Gln Glu Trp Asn Ala Arg Gln Arg Arg Pro Arg  
 1 5 10 15

Ser Leu Glu Thr Val Arg Arg Trp Val Arg Glu Cys Arg Ile Phe Pro  
 20 25 30

Pro Pro Val Lys Asp Gly Arg Glu Tyr Leu Phe His Glu Ser Ala Val  
 35 40 45

Lys Val Asp Leu Asn Arg Pro Val Thr Gly Gly Leu Leu Lys Arg Ile  
 50 55 60

Arg Asn Gly Lys Lys Ala Lys Ser  
 65 70

<210> SEQ ID NO 17  
 <211> LENGTH: 40  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence

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<220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P1  
  
 <400> SEQUENCE: 17  
  
 ctagtaagat cttgaagcct gcttttttat actaagttgg 40

<210> SEQ ID NO 18  
 <211> LENGTH: 41  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P2  
  
 <400> SEQUENCE: 18  
  
 atgatcgaat tcgaaatcaa ataatgattt tattttgact g 41

<210> SEQ ID NO 19  
 <211> LENGTH: 41  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P3  
  
 <400> SEQUENCE: 19  
  
 atgccactgc agtctgttac aggtcactaa taccatctaa g 41

<210> SEQ ID NO 20  
 <211> LENGTH: 46  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P4  
  
 <400> SEQUENCE: 20  
  
 accgttaagc tttctagacg ctcaagttag tataaaaaag ctgaac 46

<210> SEQ ID NO 21  
 <211> LENGTH: 38  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P5  
  
 <400> SEQUENCE: 21  
  
 ttcttagacg tcaggtggca cttttcgggg aaatgtgc 38

<210> SEQ ID NO 22  
 <211> LENGTH: 37  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P6  
  
 <400> SEQUENCE: 22  
  
 taacagagat ctgcgcgaga aaaaaaggat ctcaaga 37

<210> SEQ ID NO 23  
 <211> LENGTH: 46  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P7  
  
 <400> SEQUENCE: 23  
  
 aacagagatc taagcttaga tcctttgcct ggcggcagta gcgcgg 46

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<210> SEQ ID NO 24  
 <211> LENGTH: 35  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P8  
  
 <400> SEQUENCE: 24  
  
 ataaaactgca gcaaaaagag tttgtagaaa cgcaa 35

<210> SEQ ID NO 25  
 <211> LENGTH: 36  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P9  
  
 <400> SEQUENCE: 25  
  
 agtaattcta gaaagcttaa cacagaaaaa agcccg 36

<210> SEQ ID NO 26  
 <211> LENGTH: 43  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P10  
  
 <400> SEQUENCE: 26  
  
 ctagtaggat ccctgcagtg gtcgaaaaaa aaagcccgca ctg 43

<210> SEQ ID NO 27  
 <211> LENGTH: 37  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P11  
  
 <400> SEQUENCE: 27  
  
 atcgaggtag cagatctccg gataagtaga cagcctg 37

<210> SEQ ID NO 28  
 <211> LENGTH: 32  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotide P12  
  
 <400> SEQUENCE: 28  
  
 gaaggtctag agcgcccgtg tgacgctgct ag 32

<210> SEQ ID NO 29  
 <211> LENGTH: 1388  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: cloned DNA fragment EcoRI-PstI including gene  
 for tetracycline resistance (small EcoRI-Van91I fragment of  
 pBR322) and transcription terminator ter\_thrL  
  
 <400> SEQUENCE: 29  
  
 gaattctcat gtttgacagc ttatcatcga taagctttaa tgcggtagtt tatcacagtt 60  
 aaattgctaa cgcagtcagg caccgtgtat gaaatctaac aatgcgctca tcgtcatcct 120  
 cggcacgctc accctggatg ctgtaggcat aggcttggtt atgccggtac tgccgggcct 180  
 cttgcgggat atcgtccatt ccgacagcat cgccagtcac tatggcgtgc tgctagcgtc 240  
 atatgcggtg atgcaatttc tatgcgcacc cgttctcgga gcaactgtccg accgctttgg 300

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ccgccgcccc gtccctgctcg ctccgctact tggagccact atcgactacg cgatcatggc 360
gaccacacccc gtccctgtgga tcctctacgc cggacgcatac gtggcccgga tcaccggcgc 420
cacaggtgcg gttgctggcg cctatatcgc cgacatcacc gatggggaag atcgggctcg 480
ccacttcggg ctcctgagcg cttgtttcgg cgtgggtatg gtggcaggcc ccgtggccgg 540
gggactggtg ggcgccatct ccttgcatgc accattcctt gcggcggcgg tgctcaacgg 600
cctcaacctc ctactgggct gcttcctaata gcaggagtcg cataagggag agcgtcgacc 660
gatgcccttg agagccttca acccagtcag ctcttccgg tgggcgcggg gcatgactat 720
cgtcgccgca cttatgactg tcttctttat catgcaactc gtaggacagg tgccggcagc 780
gctctgggtc attttcggcg aggaccgctt tcgctggagc gcgacgatga tcggcctgctc 840
gcttgccgga ttcggaatct tgcacgcctt cgctcaagcc ttcgctactg gtcccggcac 900
caaacgtttc ggcgagaagc aggccattat cgcggcgcagc gcggccgacg cgctgggcta 960
cgtcttgctg gcgcttcgca cgcgaggctg gatggccttc cccattatga ttcttctcgc 1020
ttccggcggc atcgggatgc ccgcgttgca ggccatgctg tccaggcagg tagatgacga 1080
ccatcagggc cagcttcaag gatcgcctgc ggctcttacc agcctaactt cgatcactgg 1140
accgctgata gtcacggcga tttatgccgc ctccggcagc acatggaacg ggttgccatg 1200
gattgtaggc gccgcctat accttgctc cctccccgcg ttgcgctcgc gtgcatggag 1260
ccgggcccacc tcgacctgaa tggaagcccg cggcacctcg ctaacggatt caccactcca 1320
actagaaagc ttaacacaga aaaaagcccg cacctgacag tgcgggcttt ttttttcgac 1380
cactgcag 1388

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&lt;210&gt; SEQ ID NO 30

&lt;211&gt; LENGTH: 1162

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

```

<223> OTHER INFORMATION: cloned DNA fragment containing Artificial
Sequence DNA fragment including promoter PA2 (early promoter of
phage T7), cat gene for chloramphenicol resistance (CmR),
transcription terminator ter_thrL and attR

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&lt;400&gt; SEQUENCE: 30

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agatctccgg ataagtagac agcctgataa gtcgcacgaa aaacaggtat tgacaacatg 60
aagtaacatg cagtaagata caaatcgcta ggtaacacta gcagcgtcaa ccgggcgctc 120
tagctagagc caagctagct tggccggatc cgagattttc aggagctaag gaagctaaaa 180
tgagaaaaaa aatcactgga tataccaccg ttgatatac ccaatggcat cgtaaagaac 240
atthtgaggc atthcagtca gttgctcaat gtacctataa ccagaccgtt cagctggata 300
ttacggcctt tttaaagacc gtaaagaaaa ataagcacia gttttatccg gcctttattc 360
acattcttgc ccgcctgatg aatgctcacc cgggaattccg tatggcaatg aaagacggtg 420
agctggatgat atgggatagt gttcaccctt gttacaccgt tttccatgag caaactgaaa 480
cgttttcacc gctctggagt gaataccacg acgatttccg gcagtttcta cacatatatt 540
cgcaagatgt ggcgtgttac ggtgaaaacc tggcctattt ccctaaaggg tttattgaga 600
atatgttttt cgtctcagcc aatccctggg tgagtttcac cagttttgat ttaaactggtg 660
ccaatatgga caacttcttc gccccgctt tcaccatggg caaatattat acgcaaggcg 720
acaaggtgct gatgccgctg gcgattcagg ttcacatgac cgtctgtgat ggcttccatg 780
tcggcagaat gcttaatgaa ttacaacagt actgcgatga gtggcagggc ggggcgtaat 840
tttttaagg cagttattgg tgccctaaa cgctgggtgc tacgcctgaa taagtgataa 900

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taagcggatg aatggcagaa attcgtcgaa gcttaacaca gaaaaaagcc cgcacctgac 960
agtgcgggct ttttttttcg accactgcag tctgttacag gtcactaata ccatctaagt 1020
agttgattca tagtgactgc atatgttggtg ttttacagta ttatgtagtc tgttttttat 1080
gcaaaatcta atttaataata ttgatattta tatcatttta cgtttctcgt tcagcttttt 1140
tatactaact tgagcgtcta ga 1162

```

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<210> SEQ ID NO 31
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotide P1'

<400> SEQUENCE: 31

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ctaataatcga tgaagattct tgctcaa 27

```

```

<210> SEQ ID NO 32
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotide P2'

<400> SEQUENCE: 32

```

```

gcggtgaatt ccatacaacc tccttagtac atgc 34

```

```

<210> SEQ ID NO 33
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotide P3'

<400> SEQUENCE: 33

```

```

gtactagaat tcgtgtaatt gcgagactt tgcg 34

```

```

<210> SEQ ID NO 34
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotide P4'

<400> SEQUENCE: 34

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```

aatagcctgc agttatttga tttcaatttt gtcccactcc c 41

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```

<210> SEQ ID NO 35
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotide P5'

<400> SEQUENCE: 35

```

```

ttcttagacg tcaggtggca cttttcgggg aaatgtgc 38

```

```

<210> SEQ ID NO 36
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotide P6'

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<400> SEQUENCE: 36

taacagagat ctagcgcaga aaaaaaggat ctcaaga	37
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<210> SEQ ID NO 37

<211> LENGTH: 35

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotide P7'

<400> SEQUENCE: 37

ataaactgca gcaaaaagag tttgtagaaa cgcaa	35
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<210> SEQ ID NO 38

<211> LENGTH: 34

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotide P8'

<400> SEQUENCE: 38

aacagaagct tttgcttg cggcagtagc gcgg	34
-------------------------------------	----

<210> SEQ ID NO 39

<211> LENGTH: 879

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: cloned DNA fragment containing cI repressor gene and promoter regions

<400> SEQUENCE: 39

tcgatgaaga ttcttgctca attggtatca gctatgcgcc gaccagaaca ccttgccgat	60
---	----

cagccaaacg tctcttcagg ccaactgacta gcgataactt tccccacaac ggaacaactc	120
--	-----

tcattgcatg ggatcattgg gtactgtggg tttagtgggt gtaaaaacac ctgaccgcta	180
---	-----

tcctgatca gtttcttgaa ggtaactca tcacccccaa gtctggctat gcagaaatca	240
---	-----

cctggctcaa cagcctgctc aggtcaacg agaattaaca ttccgtcagg aaagcttggc	300
--	-----

ttggagcctg ttggtgcggt catggaatta ccttcaacct caagccagaa tgcagaatca	360
---	-----

ctggcttttt tggttgtgct tacccatctc tccgcatcac ctttggtaaa ggttctaagc	420
---	-----

tcaggtgaga acatccctgc ctgaacatga gaaaaaacag ggtactcata ctcaacttcta	480
--	-----

agtgaaggct gcatactaac cgcttcatac atctcgtaga tttctctggc gattgaaggg	540
---	-----

ctaaattctt caacgctaac tttgagaatt tttgcaagca atgcggcgtt ataagcattt	600
---	-----

aatgcattga tgccattaaa taaagcacca acgcctgact gccccatccc catcttgtct	660
---	-----

gcgacagatt cctgggataa gccaaagtca tttttctttt tttcataaat tgctttaagg	720
---	-----

cgacgtgcgt cctcaagctg ctcttggtgtt aatggtttct tttttgtgct catacgttaa	780
--	-----

atctatcacc gcaaggata aatatctaac accgtgcgtg ttgactatct tacctctggc	840
--	-----

ggtgataatg gttgcatgta ctaaggaggt tgtatggaa	879
--	-----

<210> SEQ ID NO 40

<211> LENGTH: 1290

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: cloned DNA fragment containing int-xis genes

-continued

&lt;400&gt; SEQUENCE: 40

```

attatttgat ttcaattttg tcccactccc tgcctctgtc atcacgatac tgtgatgcca      60
tgggtgtccga cttatgcccg agaagatggt gagcaaaactt atcgcttatac tgctttctcat  120
agagtctttgc agacaaaactg cgcaactcgt gaaaggtagg cggatcccct tcgaaggaaa  180
gacctgatgc ttttcgtgcg cgcataaaat accttgatac tgtgcccggat gaaagcgggt  240
cgcgacgagt agatgcaatt atggttttctc cgccaagaat ctctttgcat ttatcaagtg  300
tttccttcat tgatattccg agagcatcaa tatgcaatgc tgttgggatg gcaattttta  360
cgctgtttt gctttgctcg acataaagat atccatctac gatatacagac cacttcattt  420
cgcataaatc accaactcgt tgcccggtaa caacagccag ttccattgca agtctgagcc  480
aacatggtga tgattctgct gcttgataaa ttttcaggta ttcgtcagcc gtaagtcttg  540
atctccttac ctctgatttt gctgcgcgag tggcagcgac atggtttgtt gttatatggc  600
cttcagctat tgctctcgg aatgcatcgc tcagtgttga tctgattaac ttggctgacg  660
ccgcttgcc ctcgtctatg tatccattga gcattgccgc aatttctttt gtggtgatgt  720
cttcaagtgg agcatcaggc agaccctcc ttattgcttt aattttgctc atgtaattta  780
tgagtgtctt ctgcttgatt cctctgctgg ccaggatttt ttcgtagcga tcaagccatg  840
aatgtaacgt aacggaatta tcaactgttga ttctcgctgt cagaggcttg tgtttgtgtc  900
ctgaaaataa ctcaatgttg gcctgtatag cttcagtgat tgcgattcgc ctgtctctgc  960
ctaatccaaa ctctttaccg gtccctgggt cctgtagca gtaatatcca ttgtttctta  1020
tataaagggt agggggtaaa tcccggcgt catgacttcg cttcttccc atttctgatc  1080
ctcttcaaaa ggccacctgt tactggtcga ttaagtcaa cctttaccgc tgattcgtgg  1140
aacagatact ctcttccatc ctttaaccgga ggtgggaata tcctgcattc ccgaacccat  1200
cgacgaactg tttcaaggct tcttgacgt cgctggcgtg cgttccactc ctgaagtgtc  1260
aagtacatcg caaagtctcc gcaattacac                                     1290

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&lt;210&gt; SEQ ID NO 41

&lt;211&gt; LENGTH: 351

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: ter\_rrnB fragment (complement)

&lt;400&gt; SEQUENCE: 41

```

caaaaagagt ttgtagaac gcaaaaaggc catccgtcag gatggccttc tgettaattt      60
gatgcctggc agtttatggc gggcgtcctg cccgccacc cccgggccgt tgcttcgcaa  120
cgttcaaatc cgctcccggc ggatttgtcc tactcaggag agcgttcacc gacaaacaac  180
agataaaacg aaaggcccag tctttcgact gagcctttcg ttttatttga tgcttggcag  240
ttccctactc tcgcatgggg agaccccaca ctaccatcgg cgctacggcg tttcacttct  300
gagttcggca tggggtcagg tgggaccacc gcgctactgc cgccaggcaa a          351

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&lt;210&gt; SEQ ID NO 42

&lt;211&gt; LENGTH: 2148

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Escherichia coli

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: CDS

&lt;222&gt; LOCATION: (1)..(2148)

-continued

&lt;400&gt; SEQUENCE: 42

atg aac gtt att gca ata ttg aat cac atg ggg gtt tat ttt aaa gaa	48
Met Asn Val Ile Ala Ile Leu Asn His Met Gly Val Tyr Phe Lys Glu	
1 5 10 15	
gaa ccc atc cgt gaa ctt cat cgc gcg ctt gaa cgt ctg aac ttc cag	96
Glu Pro Ile Arg Glu Leu His Arg Ala Leu Glu Arg Leu Asn Phe Gln	
20 25 30	
att gtt tac ccg aac gac cgt gac gac tta tta aaa ctg atc gaa aac	144
Ile Val Tyr Pro Asn Asp Arg Asp Asp Leu Leu Lys Leu Ile Glu Asn	
35 40 45	
aat gcg cgt ctg tgc ggc gtt att ttt gac tgg gat aaa tat aat ctc	192
Asn Ala Arg Leu Cys Gly Val Ile Phe Asp Trp Asp Lys Tyr Asn Leu	
50 55 60	
gag ctg tgc gaa gaa att agc aaa atg aac gag aac ctg ccg ttg tac	240
Glu Leu Cys Glu Glu Ile Ser Lys Met Asn Glu Asn Leu Pro Leu Tyr	
65 70 75 80	
gcg ttc gct aat acg tat tcc act ctc gat gta agc ctg aat gac ctg	288
Ala Phe Ala Asn Thr Tyr Ser Thr Leu Asp Val Ser Leu Asn Asp Leu	
85 90 95	
cgt tta cag att agc ttc ttt gaa tat gcg ctg ggt gct gct gaa gat	336
Arg Leu Gln Ile Ser Phe Phe Glu Tyr Ala Leu Gly Ala Ala Glu Asp	
100 105 110	
att gct aat aag atc aag cag acc act gac gaa tat atc aac act att	384
Ile Ala Asn Lys Ile Lys Gln Thr Thr Asp Glu Tyr Ile Asn Thr Ile	
115 120 125	
ctg cct ccg ctg act aaa gca ctg ttt aaa tat gtt cgt gaa ggt aaa	432
Leu Pro Pro Leu Thr Lys Ala Leu Phe Lys Tyr Val Arg Glu Gly Lys	
130 135 140	
tat act ttc tgt act cct ggt cac atg ggc ggt act gca ttc cag aaa	480
Tyr Thr Phe Cys Thr Pro Gly His Met Gly Gly Thr Ala Phe Gln Lys	
145 150 155 160	
agc ccg gta ggt agc ctg ttc tat gat ttc ttt ggt ccg aat acc atg	528
Ser Pro Val Gly Ser Leu Phe Tyr Asp Phe Phe Gly Pro Asn Thr Met	
165 170 175	
aaa tct gat att tcc att tca gta tct gaa ctg ggt tct ctg ctg gat	576
Lys Ser Asp Ile Ser Ile Ser Val Ser Glu Leu Gly Ser Leu Leu Asp	
180 185 190	
cac agt ggt cca cac aaa gaa gca gaa cag tat atc gct cgc gtc ttt	624
His Ser Gly Pro His Lys Glu Ala Glu Gln Tyr Ile Ala Arg Val Phe	
195 200 205	
aac gca gac cgc agc tac atg gtg acc aac ggt act tcc act gcg aac	672
Asn Ala Asp Arg Ser Tyr Met Val Thr Asn Gly Thr Ser Thr Ala Asn	
210 215 220	
aaa att gtt ggt atg tac tct gct cca gca ggc agc acc att ctg att	720
Lys Ile Val Gly Met Tyr Ser Ala Pro Ala Gly Ser Thr Ile Leu Ile	
225 230 235 240	
gac cgt aac tgc cac aaa tcg ctg acc cac ctg atg atg atg agc gat	768
Asp Arg Asn Cys His Lys Ser Leu Thr His Leu Met Met Met Ser Asp	
245 250 255	
gtt acg cca atc tat ttc cgc ccg acc cgt aac gct tac ggt att ctt	816
Val Thr Pro Ile Tyr Phe Arg Pro Thr Arg Asn Ala Tyr Gly Ile Leu	
260 265 270	
ggt ggt atc cca cag agt gaa ttc cag cac gct acc att gct aag cgc	864
Gly Gly Ile Pro Gln Ser Glu Phe Gln His Ala Thr Ile Ala Lys Arg	
275 280 285	
gtg aaa gaa aca cca aac gca acc tgg ccg gta cat gct gta att acc	912
Val Lys Glu Thr Pro Asn Ala Thr Trp Pro Val His Ala Val Ile Thr	
290 295 300	

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aac tct acc tat gat ggt ctg ctg tac aac acc gac ttc atc aag aaa Asn Ser Thr Tyr Asp Gly Leu Leu Tyr Asn Thr Asp Phe Ile Lys Lys 305 310 315 320	960
aca ctg gat gtg aaa tcc atc cac ttt gac tcc gcg tgg gtg cct tac Thr Leu Asp Val Lys Ser Ile His Phe Asp Ser Ala Trp Val Pro Tyr 325 330 335	1008
acc aac ttc tca ccg att tac gaa ggt aaa tgc ggt atg agc ggt ggc Thr Asn Phe Ser Pro Ile Tyr Glu Gly Lys Cys Gly Met Ser Gly Gly 340 345 350	1056
cgt gta gaa ggg aaa gtg att tac gaa acc cag tcc act cac aaa ctg Arg Val Glu Gly Lys Val Ile Tyr Glu Thr Gln Ser Thr His Lys Leu 355 360 365	1104
ctg gcg gcg ttc tct cag gct tcc atg atc cac gtt aaa ggt gac gta Leu Ala Ala Phe Ser Gln Ala Ser Met Ile His Val Lys Gly Asp Val 370 375 380	1152
aac gaa gaa acc ttt aac gaa gcc tac atg atg cac acc acc act tct Asn Glu Glu Thr Phe Asn Glu Ala Tyr Met Met His Thr Thr Thr Ser 385 390 395 400	1200
ccg cac tac ggt atc gtg gcg tcc act gaa acc gct gcg gcg atg atg Pro His Tyr Gly Ile Val Ala Ser Thr Glu Thr Ala Ala Ala Met Met 405 410 415	1248
aaa ggc aat gca ggt aag cgt ctg atc aac ggt tct att gaa cgt gcg Lys Gly Asn Ala Gly Lys Arg Leu Ile Asn Gly Ser Ile Glu Arg Ala 420 425 430	1296
atc aaa ttc cgt aaa gag atc aaa cgt ctg aga acg gaa tct gat ggc Ile Lys Phe Arg Lys Glu Ile Lys Arg Leu Arg Thr Glu Ser Asp Gly 435 440 445	1344
tgg ttc ttt gat gta tgg cag ccg gat cat atc gat acg act gaa tgc Trp Phe Phe Asp Val Trp Gln Pro Asp His Ile Asp Thr Thr Glu Cys 450 455 460	1392
tgg ccg ctg cgt tct gac agc acc tgg cac ggc ttc aaa aac atc gat Trp Pro Leu Arg Ser Asp Ser Thr Trp His Gly Phe Lys Asn Ile Asp 465 470 475 480	1440
aac gag cac atg tat ctt gac ccg atc aaa gtc acc ctg ctg act ccg Asn Glu His Met Tyr Leu Asp Pro Ile Lys Val Thr Leu Leu Thr Pro 485 490 495	1488
ggg atg gaa aaa gac ggc acc atg agc gac ttt ggt att ccg gcc agc Gly Met Glu Lys Asp Gly Thr Met Ser Asp Phe Gly Ile Pro Ala Ser 500 505 510	1536
atc gtg gcg aaa tac ctc gac gaa cat ggc atc gtt gtt gag aaa acc Ile Val Ala Lys Tyr Leu Asp Glu His Gly Ile Val Val Glu Lys Thr 515 520 525	1584
ggt ccg tat aac ctg ctg ttc ctg ttc agc atc ggt atc gat aag acc Gly Pro Tyr Asn Leu Leu Phe Leu Phe Ser Ile Gly Ile Asp Lys Thr 530 535 540	1632
aaa gca ctg agc ctg ctg cgt gct ctg act gac ttt aaa cgt gcg ttc Lys Ala Leu Ser Leu Leu Arg Ala Leu Thr Asp Phe Lys Arg Ala Phe 545 550 555 560	1680
gac ctg aac ctg cgt gtg aaa aac atg ctg ccg tct ctg tat cgt gaa Asp Leu Asn Leu Arg Val Lys Asn Met Leu Pro Ser Leu Tyr Arg Glu 565 570 575	1728
gat cct gaa ttc tat gaa aac atg cgt att cag gaa ctg gct cag aat Asp Pro Glu Phe Tyr Glu Asn Met Arg Ile Gln Glu Leu Ala Gln Asn 580 585 590	1776
atc cac aaa ctg att gtt cac cac aat ctg ccg gat ctg atg tat cgc Ile His Lys Leu Ile Val His His Asn Leu Pro Asp Leu Met Tyr Arg 595 600 605	1824
gca ttt gaa gtg ctg ccg acg atg gta atg act ccg tat gct gca ttc Ala Phe Glu Val Leu Pro Thr Met Val Met Thr Pro Tyr Ala Ala Phe 610 615 620	1872

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cag	aaa	gag	ctg	cac	ggt	atg	acc	gaa	gaa	ggt	tac	ctc	gac	gaa	atg	1920
Gln	Lys	Glu	Leu	His	Gly	Met	Thr	Glu	Glu	Val	Tyr	Leu	Asp	Glu	Met	
625					630					635				640		
gta	ggt	cgt	att	aac	gcc	aat	atg	atc	ctt	ccg	tac	ccg	ccg	gga	ggt	1968
Val	Gly	Arg	Ile	Asn	Ala	Asn	Met	Ile	Leu	Pro	Tyr	Pro	Pro	Gly	Val	
				645					650					655		
cct	ctg	gta	atg	ccg	ggt	gaa	atg	atc	acc	gaa	gaa	agc	cgt	ccg	ggt	2016
Pro	Leu	Val	Met	Pro	Gly	Glu	Met	Ile	Thr	Glu	Glu	Ser	Arg	Pro	Val	
			660					665					670			
ctg	gag	ttc	ctg	cag	atg	ctg	tgt	gaa	atc	ggc	gct	cac	tat	ccg	ggc	2064
Leu	Glu	Phe	Leu	Gln	Met	Leu	Cys	Glu	Ile	Gly	Ala	His	Tyr	Pro	Gly	
		675					680					685				
ttt	gaa	acc	gat	att	cac	ggt	gca	tac	cgt	cag	gct	gat	ggc	cgc	tat	2112
Phe	Glu	Thr	Asp	Ile	His	Gly	Ala	Tyr	Arg	Gln	Ala	Asp	Gly	Arg	Tyr	
	690					695					700					
acc	ggt	aag	gta	ttg	aaa	gaa	gaa	agc	aaa	aaa	taa					2148
Thr	Val	Lys	Val	Leu	Lys	Glu	Glu	Ser	Lys	Lys						
705					710					715						

&lt;210&gt; SEQ ID NO 43

&lt;211&gt; LENGTH: 715

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Escherichia coli

&lt;400&gt; SEQUENCE: 43

Met	Asn	Val	Ile	Ala	Ile	Leu	Asn	His	Met	Gly	Val	Tyr	Phe	Lys	Glu	
1				5					10					15		
Glu	Pro	Ile	Arg	Glu	Leu	His	Arg	Ala	Leu	Glu	Arg	Leu	Asn	Phe	Gln	
			20					25					30			
Ile	Val	Tyr	Pro	Asn	Asp	Arg	Asp	Asp	Leu	Leu	Lys	Leu	Ile	Glu	Asn	
		35					40					45				
Asn	Ala	Arg	Leu	Cys	Gly	Val	Ile	Phe	Asp	Trp	Asp	Lys	Tyr	Asn	Leu	
	50				55					60						
Glu	Leu	Cys	Glu	Glu	Ile	Ser	Lys	Met	Asn	Glu	Asn	Leu	Pro	Leu	Tyr	
65					70					75					80	
Ala	Phe	Ala	Asn	Thr	Tyr	Ser	Thr	Leu	Asp	Val	Ser	Leu	Asn	Asp	Leu	
				85					90					95		
Arg	Leu	Gln	Ile	Ser	Phe	Phe	Glu	Tyr	Ala	Leu	Gly	Ala	Ala	Glu	Asp	
		100						105					110			
Ile	Ala	Asn	Lys	Ile	Lys	Gln	Thr	Thr	Asp	Glu	Tyr	Ile	Asn	Thr	Ile	
		115					120					125				
Leu	Pro	Pro	Leu	Thr	Lys	Ala	Leu	Phe	Lys	Tyr	Val	Arg	Glu	Gly	Lys	
	130					135					140					
Tyr	Thr	Phe	Cys	Thr	Pro	Gly	His	Met	Gly	Gly	Thr	Ala	Phe	Gln	Lys	
145					150					155					160	
Ser	Pro	Val	Gly	Ser	Leu	Phe	Tyr	Asp	Phe	Phe	Gly	Pro	Asn	Thr	Met	
				165					170					175		
Lys	Ser	Asp	Ile	Ser	Ile	Ser	Val	Ser	Glu	Leu	Gly	Ser	Leu	Leu	Asp	
			180					185					190			
His	Ser	Gly	Pro	His	Lys	Glu	Ala	Glu	Gln	Tyr	Ile	Ala	Arg	Val	Phe	
		195					200					205				
Asn	Ala	Asp	Arg	Ser	Tyr	Met	Val	Thr	Asn	Gly	Thr	Ser	Thr	Ala	Asn	
	210					215					220					
Lys	Ile	Val	Gly	Met	Tyr	Ser	Ala	Pro	Ala	Gly	Ser	Thr	Ile	Leu	Ile	
225					230					235					240	
Asp	Arg	Asn	Cys	His	Lys	Ser	Leu	Thr	His	Leu	Met	Met	Met	Ser	Asp	
				245					250						255	

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Val	Thr	Pro	Ile	Tyr	Phe	Arg	Pro	Thr	Arg	Asn	Ala	Tyr	Gly	Ile	Leu
			260					265					270		
Gly	Gly	Ile	Pro	Gln	Ser	Glu	Phe	Gln	His	Ala	Thr	Ile	Ala	Lys	Arg
		275					280					285			
Val	Lys	Glu	Thr	Pro	Asn	Ala	Thr	Trp	Pro	Val	His	Ala	Val	Ile	Thr
	290					295					300				
Asn	Ser	Thr	Tyr	Asp	Gly	Leu	Leu	Tyr	Asn	Thr	Asp	Phe	Ile	Lys	Lys
305					310					315					320
Thr	Leu	Asp	Val	Lys	Ser	Ile	His	Phe	Asp	Ser	Ala	Trp	Val	Pro	Tyr
				325					330					335	
Thr	Asn	Phe	Ser	Pro	Ile	Tyr	Glu	Gly	Lys	Cys	Gly	Met	Ser	Gly	Gly
			340					345					350		
Arg	Val	Glu	Gly	Lys	Val	Ile	Tyr	Glu	Thr	Gln	Ser	Thr	His	Lys	Leu
		355					360					365			
Leu	Ala	Ala	Phe	Ser	Gln	Ala	Ser	Met	Ile	His	Val	Lys	Gly	Asp	Val
	370					375					380				
Asn	Glu	Glu	Thr	Phe	Asn	Glu	Ala	Tyr	Met	Met	His	Thr	Thr	Thr	Ser
385					390					395					400
Pro	His	Tyr	Gly	Ile	Val	Ala	Ser	Thr	Glu	Thr	Ala	Ala	Ala	Met	Met
				405					410					415	
Lys	Gly	Asn	Ala	Gly	Lys	Arg	Leu	Ile	Asn	Gly	Ser	Ile	Glu	Arg	Ala
			420					425					430		
Ile	Lys	Phe	Arg	Lys	Glu	Ile	Lys	Arg	Leu	Arg	Thr	Glu	Ser	Asp	Gly
		435					440					445			
Trp	Phe	Phe	Asp	Val	Trp	Gln	Pro	Asp	His	Ile	Asp	Thr	Thr	Glu	Cys
	450					455					460				
Trp	Pro	Leu	Arg	Ser	Asp	Ser	Thr	Trp	His	Gly	Phe	Lys	Asn	Ile	Asp
465					470					475					480
Asn	Glu	His	Met	Tyr	Leu	Asp	Pro	Ile	Lys	Val	Thr	Leu	Leu	Thr	Pro
				485					490					495	
Gly	Met	Glu	Lys	Asp	Gly	Thr	Met	Ser	Asp	Phe	Gly	Ile	Pro	Ala	Ser
			500					505					510		
Ile	Val	Ala	Lys	Tyr	Leu	Asp	Glu	His	Gly	Ile	Val	Val	Glu	Lys	Thr
		515					520					525			
Gly	Pro	Tyr	Asn	Leu	Leu	Phe	Leu	Phe	Ser	Ile	Gly	Ile	Asp	Lys	Thr
	530					535					540				
Lys	Ala	Leu	Ser	Leu	Leu	Arg	Ala	Leu	Thr	Asp	Phe	Lys	Arg	Ala	Phe
545						550				555					560
Asp	Leu	Asn	Leu	Arg	Val	Lys	Asn	Met	Leu	Pro	Ser	Leu	Tyr	Arg	Glu
			565						570					575	
Asp	Pro	Glu	Phe	Tyr	Glu	Asn	Met	Arg	Ile	Gln	Glu	Leu	Ala	Gln	Asn
			580					585					590		
Ile	His	Lys	Leu	Ile	Val	His	His	Asn	Leu	Pro	Asp	Leu	Met	Tyr	Arg
		595					600					605			
Ala	Phe	Glu	Val	Leu	Pro	Thr	Met	Val	Met	Thr	Pro	Tyr	Ala	Ala	Phe
	610					615					620				
Gln	Lys	Glu	Leu	His	Gly	Met	Thr	Glu	Glu	Val	Tyr	Leu	Asp	Glu	Met
625					630					635					640
Val	Gly	Arg	Ile	Asn	Ala	Asn	Met	Ile	Leu	Pro	Tyr	Pro	Pro	Gly	Val
				645					650					655	
Pro	Leu	Val	Met	Pro	Gly	Glu	Met	Ile	Thr	Glu	Glu	Ser	Arg	Pro	Val
			660					665					670		
Leu	Glu	Phe	Leu	Gln	Met	Leu	Cys	Glu	Ile	Gly	Ala	His	Tyr	Pro	Gly
		675					680					685			

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Phe Glu Thr Asp Ile His Gly Ala Tyr Arg Gln Ala Asp Gly Arg Tyr  
690 695 700

Thr Val Lys Val Leu Lys Glu Glu Ser Lys Lys  
705 710 715

<210> SEQ ID NO 44  
<211> LENGTH: 2142  
<212> TYPE: DNA  
<213> ORGANISM: Escherichia coli  
<220> FEATURE:  
<221> NAME/KEY: CDS  
<222> LOCATION: (1)..(2142)

<400> SEQUENCE: 44

atg aac atc att gcc att atg gga cgc cat ggc gtc ttt tat aaa gat	48
Met Asn Ile Ile Ala Ile Met Gly Pro His Gly Val Phe Tyr Lys Asp	
1 5 10 15	
gag ccc atc aaa gaa ctg gag tcg gcg ctg gtg gcg caa ggc ttt cag	96
Glu Pro Ile Lys Glu Leu Glu Ser Ala Leu Val Ala Gln Gly Phe Gln	
20 25 30	
att atc tgg cca caa aac agc gtt gat ttg ctg aaa ttt atc gag cat	144
Ile Ile Trp Pro Gln Asn Ser Val Asp Leu Leu Lys Phe Ile Glu His	
35 40 45	
aac cct cga att tgc ggc gtg att ttt gac tgg gat gag tac agt ctc	192
Asn Pro Arg Ile Cys Gly Val Ile Phe Asp Trp Asp Glu Tyr Ser Leu	
50 55 60	
gat tta tgt agc gat atc aat cag ctt aat gaa tat ctc ccg ctt tat	240
Asp Leu Cys Ser Asp Ile Asn Gln Leu Asn Glu Tyr Leu Pro Leu Tyr	
65 70 75 80	
gcc ttc atc aac acc cac tcg acg atg gat gtc agc gtg cag gat atg	288
Ala Phe Ile Asn Thr His Ser Thr Met Asp Val Ser Val Gln Asp Met	
85 90 95	
cgg atg gcg ctc tgg ttt ttt gaa tat gcg ctg ggg cag gcg gaa gat	336
Arg Met Ala Leu Trp Phe Phe Glu Tyr Ala Leu Gly Gln Ala Glu Asp	
100 105 110	
atc gcc att cgt atg cgt cag tac acc gac gaa tat ctt gat aac att	384
Ile Ala Ile Arg Met Arg Gln Tyr Thr Asp Glu Tyr Leu Asp Asn Ile	
115 120 125	
aca ccg ccg ttc acg aaa gcc ttg ttt acc tac gtc aaa gag ccg aag	432
Thr Pro Pro Phe Thr Lys Ala Leu Phe Thr Tyr Val Lys Glu Arg Lys	
130 135 140	
tac acc ttt tgt acg ccg ggg cat atg ggc ggc acc gca tat caa aaa	480
Tyr Thr Phe Cys Thr Pro Gly His Met Gly Gly Thr Ala Tyr Gln Lys	
145 150 155 160	
agc ccg gtt ggc tgt ctg ttt tat gat ttt ttc ggc ggc aat act ctt	528
Ser Pro Val Gly Cys Leu Phe Tyr Asp Phe Phe Gly Gly Asn Thr Leu	
165 170 175	
aag gct gat gtc tct att tcg gtc acc gag ctt ggt tcg ttg ctc gac	576
Lys Ala Asp Val Ser Ile Ser Val Thr Glu Leu Gly Ser Leu Leu Asp	
180 185 190	
cac acc ggg cca cac ctg gaa gcg gaa gag tac atc gcg ccg act ttt	624
His Thr Gly Pro His Leu Glu Ala Glu Glu Tyr Ile Ala Arg Thr Phe	
195 200 205	
ggc gcg gaa cag agt tat atc gtt acc aac gga aca tcg acg tcg aac	672
Gly Ala Glu Gln Ser Tyr Ile Val Thr Asn Gly Thr Ser Thr Ser Asn	
210 215 220	
aaa att gtg ggt atg tac gcc gcg cca tcc ggc agt acg ctg ttg atc	720
Lys Ile Val Gly Met Tyr Ala Ala Pro Ser Gly Ser Thr Leu Leu Ile	
225 230 235 240	
gac cgc aat tgt cat aaa tcg ctg gcg cat ctg ttg atg atg aac gat	768
Asp Arg Asn Cys His Lys Ser Leu Ala His Leu Leu Met Met Asn Asp	
245 250 255	



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gta gtg cca gtc tgg ctg aaa ccg acg cgt aat gcg ttg ggg att ctt	816
Val Val Pro Val Trp Leu Lys Pro Thr Arg Asn Ala Leu Gly Ile Leu	
260 265 270	
ggt ggg atc ccg cgc cgt gaa ttt act cgc gac agc atc gaa gag aaa	864
Gly Gly Ile Pro Arg Arg Glu Phe Thr Arg Asp Ser Ile Glu Glu Lys	
275 280 285	
gtc gct gct acc acg caa gca caa tgg ccg gtt cat gcg gtg atc acc	912
Val Ala Ala Thr Thr Gln Ala Gln Trp Pro Val His Ala Val Ile Thr	
290 295 300	
aac tcc acc tat gat ggc ttg ctc tac aac acc gac tgg atc aaa cag	960
Asn Ser Thr Tyr Asp Gly Leu Leu Tyr Asn Thr Asp Trp Ile Lys Gln	
305 310 315 320	
acg ctg gat gtc ccg tcg att cac ttc gat tct gcc tgg gtg ccg tac	1008
Thr Leu Asp Val Pro Ser Ile His Phe Asp Ser Ala Trp Val Pro Tyr	
325 330 335	
acc cat ttt cat ccg atc tac cag ggt aaa agt ggt atg agc ggc gag	1056
Thr His Phe His Pro Ile Tyr Gln Gly Lys Ser Gly Met Ser Gly Glu	
340 345 350	
cgt gtt gcg gga aaa gtg atc ttc gaa acg caa tcg acc cac aaa atg	1104
Arg Val Ala Gly Lys Val Ile Phe Glu Thr Gln Ser Thr His Lys Met	
355 360 365	
ctg gcg gcg tta tcg cag gct tcg ctg atc cac att aaa ggc gag tat	1152
Leu Ala Ala Leu Ser Gln Ala Ser Leu Ile His Ile Lys Gly Glu Tyr	
370 375 380	
gac gaa gag gcc ttt aac gaa gcc ttt atg atg cat acc acc acc tcg	1200
Asp Glu Glu Ala Phe Asn Glu Ala Phe Met Met His Thr Thr Thr Ser	
385 390 395 400	
ccc agt tat ccc att gtt gct tcg gtt gag acg gcg gcg gcg atg ctg	1248
Pro Ser Tyr Pro Ile Val Ala Ser Val Glu Thr Ala Ala Ala Met Leu	
405 410 415	
cgt ggt aat ccg ggc aaa cgg ctg att aac cgt tca gta gaa cga gct	1296
Arg Gly Asn Pro Gly Lys Arg Leu Ile Asn Arg Ser Val Glu Arg Ala	
420 425 430	
ctg cat ttt cgc aaa gag gtc cag cgg ctg cgg gaa gag tct gac ggt	1344
Leu His Phe Arg Lys Glu Val Gln Arg Leu Arg Glu Glu Ser Asp Gly	
435 440 445	
tgg ttt ttc gat atc tgg caa ccg ccg cag gtg gat gaa gcc gaa tgc	1392
Trp Phe Phe Asp Ile Trp Gln Pro Pro Gln Val Asp Glu Ala Glu Cys	
450 455 460	
tgg ccc gtt gcg cct ggc gaa cag tgg cac ggc ttt aac gat gcg gat	1440
Trp Pro Val Ala Pro Gly Glu Gln Trp His Gly Phe Asn Asp Ala Asp	
465 470 475 480	
gcc gat cat atg ttt ctc gat ccg gtt aaa gtc act att ttg aca ccg	1488
Ala Asp His Met Phe Leu Asp Pro Val Lys Val Thr Ile Leu Thr Pro	
485 490 495	
ggg atg gac gag cag ggc aat atg agc gag gag ggg atc ccg gcg gcg	1536
Gly Met Asp Glu Gln Gly Asn Met Ser Glu Glu Gly Ile Pro Ala Ala	
500 505 510	
ctg gta gca aaa ttc ctc gac gaa cgt ggg atc gta gta gag aaa acc	1584
Leu Val Ala Lys Phe Leu Asp Glu Arg Gly Ile Val Val Glu Lys Thr	
515 520 525	
ggc cct tat aac ctg ctg ttt ctc ttt agt att ggc atc gat aaa acc	1632
Gly Pro Tyr Asn Leu Leu Phe Leu Phe Ser Ile Gly Ile Asp Lys Thr	
530 535 540	
aaa gca atg gga tta ttg cgt ggg ttg acg gaa ttc aaa cgc tct tac	1680
Lys Ala Met Gly Leu Leu Arg Gly Leu Thr Glu Phe Lys Arg Ser Tyr	
545 550 555 560	
gat ctc aac ctg cgg atc aaa aat atg cta ccc gat ctc tat gca gaa	1728
Asp Leu Asn Leu Arg Ile Lys Asn Met Leu Pro Asp Leu Tyr Ala Glu	
565 570 575	

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gat ccc gat ttc tac cgc aat atg cgt att cag gat ctg gca caa ggg	1776
Asp Pro Asp Phe Tyr Arg Asn Met Arg Ile Gln Asp Leu Ala Gln Gly	
580 585 590	
atc cat aag ctg att cgt aaa cac gat ctt ccc ggt ttg atg ttg cgg	1824
Ile His Lys Leu Ile Arg Lys His Asp Leu Pro Gly Leu Met Leu Arg	
595 600 605	
gca ttc gat act ttg ccg gag atg atc atg acg cca cat cag gca tgg	1872
Ala Phe Asp Thr Leu Pro Glu Met Ile Met Thr Pro His Gln Ala Trp	
610 615 620	
caa cga caa att aaa ggc gaa gta gaa acc att gcg ctg gaa caa ctg	1920
Gln Arg Gln Ile Lys Gly Glu Val Glu Thr Ile Ala Leu Glu Gln Leu	
625 630 635 640	
gtc ggt aga gta tcg gca aat atg atc ctg cct tat cca ccg ggc gta	1968
Val Gly Arg Val Ser Ala Asn Met Ile Leu Pro Tyr Pro Pro Gly Val	
645 650 655	
ccg ctg ttg atg cct gga gaa atg ctg acc aaa gag agc cgc aca gta	2016
Pro Leu Leu Met Pro Gly Glu Met Leu Thr Lys Glu Ser Arg Thr Val	
660 665 670	
ctc gat ttt cta ctg atg ctt tgt tcc gtc ggg caa cat tac ccc ggt	2064
Leu Asp Phe Leu Leu Met Leu Cys Ser Val Gly Gln His Tyr Pro Gly	
675 680 685	
ttt gaa acg gat att cac ggc gcg aaa cag gac gaa gac ggc gtt tac	2112
Phe Glu Thr Asp Ile His Gly Ala Lys Gln Asp Glu Asp Gly Val Tyr	
690 695 700	
cgc gta cga gtc cta aaa atg gcg gga taa	2142
Arg Val Arg Val Leu Lys Met Ala Gly	
705 710	

&lt;210&gt; SEQ ID NO 45

&lt;211&gt; LENGTH: 713

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Escherichia coli

&lt;400&gt; SEQUENCE: 45

Met Asn Ile Ile Ala Ile Met Gly Pro His Gly Val Phe Tyr Lys Asp	
1 5 10 15	
Glu Pro Ile Lys Glu Leu Glu Ser Ala Leu Val Ala Gln Gly Phe Gln	
20 25 30	
Ile Ile Trp Pro Gln Asn Ser Val Asp Leu Leu Lys Phe Ile Glu His	
35 40 45	
Asn Pro Arg Ile Cys Gly Val Ile Phe Asp Trp Asp Glu Tyr Ser Leu	
50 55 60	
Asp Leu Cys Ser Asp Ile Asn Gln Leu Asn Glu Tyr Leu Pro Leu Tyr	
65 70 75 80	
Ala Phe Ile Asn Thr His Ser Thr Met Asp Val Ser Val Gln Asp Met	
85 90 95	
Arg Met Ala Leu Trp Phe Phe Glu Tyr Ala Leu Gly Gln Ala Glu Asp	
100 105 110	
Ile Ala Ile Arg Met Arg Gln Tyr Thr Asp Glu Tyr Leu Asp Asn Ile	
115 120 125	
Thr Pro Pro Phe Thr Lys Ala Leu Phe Thr Tyr Val Lys Glu Arg Lys	
130 135 140	
Tyr Thr Phe Cys Thr Pro Gly His Met Gly Gly Thr Ala Tyr Gln Lys	
145 150 155 160	
Ser Pro Val Gly Cys Leu Phe Tyr Asp Phe Phe Gly Gly Asn Thr Leu	
165 170 175	
Lys Ala Asp Val Ser Ile Ser Val Thr Glu Leu Gly Ser Leu Leu Asp	
180 185 190	

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His	Thr	Gly	Pro	His	Leu	Glu	Ala	Glu	Glu	Tyr	Ile	Ala	Arg	Thr	Phe
		195					200					205			
Gly	Ala	Glu	Gln	Ser	Tyr	Ile	Val	Thr	Asn	Gly	Thr	Ser	Thr	Ser	Asn
	210					215					220				
Lys	Ile	Val	Gly	Met	Tyr	Ala	Ala	Pro	Ser	Gly	Ser	Thr	Leu	Leu	Ile
225					230					235					240
Asp	Arg	Asn	Cys	His	Lys	Ser	Leu	Ala	His	Leu	Leu	Met	Met	Asn	Asp
			245						250					255	
Val	Val	Pro	Val	Trp	Leu	Lys	Pro	Thr	Arg	Asn	Ala	Leu	Gly	Ile	Leu
			260					265					270		
Gly	Gly	Ile	Pro	Arg	Arg	Glu	Phe	Thr	Arg	Asp	Ser	Ile	Glu	Glu	Lys
		275					280					285			
Val	Ala	Ala	Thr	Thr	Gln	Ala	Gln	Trp	Pro	Val	His	Ala	Val	Ile	Thr
	290					295					300				
Asn	Ser	Thr	Tyr	Asp	Gly	Leu	Leu	Tyr	Asn	Thr	Asp	Trp	Ile	Lys	Gln
305					310					315					320
Thr	Leu	Asp	Val	Pro	Ser	Ile	His	Phe	Asp	Ser	Ala	Trp	Val	Pro	Tyr
				325					330					335	
Thr	His	Phe	His	Pro	Ile	Tyr	Gln	Gly	Lys	Ser	Gly	Met	Ser	Gly	Glu
			340					345					350		
Arg	Val	Ala	Gly	Lys	Val	Ile	Phe	Glu	Thr	Gln	Ser	Thr	His	Lys	Met
		355					360					365			
Leu	Ala	Ala	Leu	Ser	Gln	Ala	Ser	Leu	Ile	His	Ile	Lys	Gly	Glu	Tyr
	370					375					380				
Asp	Glu	Glu	Ala	Phe	Asn	Glu	Ala	Phe	Met	Met	His	Thr	Thr	Thr	Ser
385					390					395					400
Pro	Ser	Tyr	Pro	Ile	Val	Ala	Ser	Val	Glu	Thr	Ala	Ala	Ala	Met	Leu
				405					410					415	
Arg	Gly	Asn	Pro	Gly	Lys	Arg	Leu	Ile	Asn	Arg	Ser	Val	Glu	Arg	Ala
			420					425					430		
Leu	His	Phe	Arg	Lys	Glu	Val	Gln	Arg	Leu	Arg	Glu	Glu	Ser	Asp	Gly
		435					440					445			
Trp	Phe	Phe	Asp	Ile	Trp	Gln	Pro	Pro	Gln	Val	Asp	Glu	Ala	Glu	Cys
	450					455					460				
Trp	Pro	Val	Ala	Pro	Gly	Glu	Gln	Trp	His	Gly	Phe	Asn	Asp	Ala	Asp
465					470					475					480
Ala	Asp	His	Met	Phe	Leu	Asp	Pro	Val	Lys	Val	Thr	Ile	Leu	Thr	Pro
				485					490					495	
Gly	Met	Asp	Glu	Gln	Gly	Asn	Met	Ser	Glu	Glu	Gly	Ile	Pro	Ala	Ala
			500					505					510		
Leu	Val	Ala	Lys	Phe	Leu	Asp	Glu	Arg	Gly	Ile	Val	Val	Glu	Lys	Thr
		515					520					525			
Gly	Pro	Tyr	Asn	Leu	Leu	Phe	Leu	Phe	Ser	Ile	Gly	Ile	Asp	Lys	Thr
	530					535					540				
Lys	Ala	Met	Gly	Leu	Leu	Arg	Gly	Leu	Thr	Glu	Phe	Lys	Arg	Ser	Tyr
545					550					555					560
Asp	Leu	Asn	Leu	Arg	Ile	Lys	Asn	Met	Leu	Pro	Asp	Leu	Tyr	Ala	Glu
			565						570					575	
Asp	Pro	Asp	Phe	Tyr	Arg	Asn	Met	Arg	Ile	Gln	Asp	Leu	Ala	Gln	Gly
			580					585					590		
Ile	His	Lys	Leu	Ile	Arg	Lys	His	Asp	Leu	Pro	Gly	Leu	Met	Leu	Arg
		595					600					605			
Ala	Phe	Asp	Thr	Leu	Pro	Glu	Met	Ile	Met	Thr	Pro	His	Gln	Ala	Trp
	610					615						620			



**81**

- (a) a DNA comprising the nucleotide sequence of SEQ ID NO. 5, and
  - (b) a DNA encoding a protein having  $\beta$ -glucoside PTS activity which hybridizes with a DNA comprising a sequence fully complementary to the nucleotide sequence of SEQ ID NO. 5 under stringent conditions comprising washing at 60° C., in a salt concentration of 1×SSC, 0.1% SDS.
3. The method according to claim 1, wherein the bglF gene encodes a protein selected from the group consisting of:

**82**

- (A) a protein comprising the amino acid sequence of SEQ ID NO.6, and
  - (B) a protein comprising an amino acid sequence which is at least 95% homologous to the amino acid sequence of SEQ ID NO.6, and has  $\beta$ -glucoside PTS activity.
4. The method according to claim 1, wherein said L-amino acid is selected from the group consisting of L-lysine, L-threonine, L-glutamic acid, and combinations thereof.

\* \* \* \* \*